Tyrosine Phosphorylation of Paxillin and pp125FAK Accompanies Cell Adhesion to Extracellular Matrix: A Role in Cytoskeletal Assembly

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Abstract. Cells in culture reveal high levels of protein tyrosine phosphorylation in their focal adhesions, the regions where cells adhere to the underlying substratum. We have examined the tyrosine phosphorylation of proteins in response to plating cells on extracellular matrix substrata. Rat embryo fibroblasts, mouse Balb/c 3T3, and NIH 3T3 cells plated on fibronectin-coated surfaces revealed elevated phosphotyrosine levels in a cluster of proteins between 115 and 130 kD. This increase in tyrosine phosphorylation was also seen when rat embryo fibroblasts were plated on laminin or vitronectin, but not on polylysine or on uncoated plastic. Integrin mediation of this effect was suggested by finding the same pattern of elevated tyrosine phosphorylation in cells plated on the cell-binding fragment of fibronectin and in cells plated on a synthetic polymer containing multiple RGD sequences. We have identified one of the proteins of the 115–130-kD cluster as pp125FAK, a tyrosine kinase recently localized in focal adhesions (Schaller, M. D., C. A. Borgman, B. S. Cobb, R. R. Vines, A. B. Reynolds, and J. T. Parsons. 1992. Proc. Natl. Acad. Sci. USA. 89:5192). A second protein that becomes tyrosine phosphorylated in response to extracellular matrix adhesion is identified as paxillin, a 70-kD protein previously localized in focal adhesions. Treatment of cells with the tyrosine kinase inhibitor herbimycin A diminished the adhesion-induced tyrosine phosphorylation of these proteins and inhibited the formation of focal adhesions and stress fibers. These results suggest a role for integrin-mediated tyrosine phosphorylation in the organization of the cytoskeleton as cells adhere to the extracellular matrix.

The extracellular matrix (ECM) affects many aspects of cell behavior, including the migratory properties of cells, their morphology, growth characteristics, and differentiation (Hay, 1981; Hynes, 1990). The means by which the ECM exerts these effects are largely unknown, but there is evidence that many of these actions are mediated through integrins, the family of ECM receptors found on most cells. Integrins are αβ heterodimers; each subunit has a large extracellular domain, spans the membrane once, and has a short cytoplasmic sequence (Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Multiple α and β subunits have been identified (Albelda and Buck, 1990; Hemler, 1990; Hynes, 1992). Some subunits are shared by many integrins (e.g., β3), whereas others appear to be unique. During the last few years much has been learned about the interaction of integrins with particular ECM components such as fibronectin or vitronectin. In many cells in culture, integrins are clustered at sites of adhesion to the underlying ECM in regions known as focal adhesions (focal contacts or adhesion plaques) (for review, see Burridge et al., 1988). The extracellular face of focal adhesions provides attachment to matrix components adsorbed onto the plastic or glass culture surface, whereas the cytoplasmic face provides a site of attachment for bundles of actin filaments (stress fibers). Evidence from in vitro binding experiments indicates that integrins can interact with at least two cytoskeletal proteins, talin (Horwitz et al., 1986) and α-actinin (Otey et al., 1990). Along with several other cytoskeletal proteins, talin and α-actinin are concentrated at the cytoplasmic face of focal adhesions (Burridge et al., 1988).

Focal adhesions are thought to be important not only as structural links between the ECM and the cytoskeleton, but also as sites of signal transduction from the ECM. Several potential regulatory enzymes have been identified in focal adhesions (Burridge et al., 1988). These include protein kinase C (Jaken et al., 1989) and several tyrosine kinases (Rohrschneider, 1980; Rohrschneider and Gentry, 1984; Rohrschneider and Najita, 1984). Considerable attention has been directed toward the tyrosine kinase pp60src, which is localized to the residual focal adhesions of cells transformed by Rous sarcoma virus (Rohrschneider, 1980). Pp60src has not yet been detected in the focal adhesions of normal cells, but elevated phosphotyrosine has been noted...
at these sites in both transformed (Comoglio et al., 1984) and normal cells (Maher et al., 1985), indicating that this or other tyrosine kinases must be present and active. The newly described tyrosine kinase, pp125FAK, has been cloned, sequenced, and localized to the focal adhesions of normal fibroblasts by Parsons' group (Schaller et al., 1992). Direct evidence for a relationship between integrins and tyrosine kinase activation has been provided by Kornberg et al. (1991) and by Guan et al. (1991). Kornberg and co-workers demonstrated an elevation in phosphotyrosine in proteins of \( \sim 130 \) kD when the integrin (\( \alpha_5 \beta_1 \)) was aggregated by antibodies on the surface of KB cells. Guan and colleagues found that plating NIH 3T3 cells on fibronectin, but not on polylysine or plastic, led to the tyrosine phosphorylation of a protein at \( \sim 120 \) kD. This phosphorylation could also be induced by adhesion of these cells to an anti-integrin antibody and it was shown that this response required the cytoplasmic domain of the \( \beta_1 \) integrin (Guan et al., 1991).

In this paper, we have explored the tyrosine phosphorylation that occurs in response to cell adhesion to different extracellular matrix substrates. We detect a prominent cluster of bands with elevated phosphotyrosine that are between 115 and 130 kD in extracts from rat embryo fibroblasts, mouse NIH 3T3, and Balb/c 3T3 cells adhering to fibronectin. Phosphorylation on tyrosine is low in these bands in extracts of NIH 3T3, and Balb/c 3T3 cells adhering to fibronectin. Phosphorylation induced by cell adhesion to fibronectin is in the same cell types that have been plated on plastic or kept in suspension. We show that one of these proteins is pp125FABK, the new tyrosine kinase identified by Schaller et al. (1992). We identify a second protein of \( \sim 70 \) kD that becomes tyrosine-phosphorylated in response to ECM adhesion as paxillin, a focal adhesion protein (Turner et al., 1991) that is also tyrosine-phosphorylated during embryonic development (Turner, 1991). The increase in tyrosine phosphorylation induced by cell adhesion to fibronectin is inhibited by herbimycin A. Cells treated with herbimycin A show a decrease in focal adhesion and stress fiber formation. These findings suggest that tyrosine phosphorylation plays a role in the cytoskeletal organization induced by integrin-mediated adhesion to ECM.

**Materials and Methods**

**Cell Culture and Adhesion to Substrates**

Rat embryo fibroblasts (REF52) and mouse Balb/c 3T3 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 \( \mu \)g/ml streptomycin. Mouse NIH 3T3 cells were cultured in the same medium with 10% calf serum. Cells were passaged 1-3 times before use. For adhesion experiments the REF52 cells were trypsinized, washed once in medium containing 10% serum, washed once in serum-free medium, resuspended, and plated in serum-free medium. For adhesion experiments with Balb/c 3T3 and NIH 3T3 cells, the cultures were pretreated with 25 \( \mu \)g/ml cycloheximide for 2 h. After trypsinization, the cells were washed once in serum-free medium and resuspended and plated in serum-free medium. For gel and blot analysis cells were plated at \( \sim 4 \times 10^6 \) cells/10-cm culture dish. Coverslips or culture dishes were coated with ECM proteins or other polymers by incubation at 37°C for 90 min, or in some experiments by incubation overnight at 4°C, except for Pronectin-F, which was incubated with surfaces for 90 min at room temperature. Before plating cells, coverslips or dishes were rinsed twice in PBS. Poly-L-lysine (Sigma Chemical Co.) was used at 0.5 mg/ml; human plasma fibronectin (New York Blood Center, New York) was used at 0.05-0.1 mg/ml; 105-kD cell-binding fragment of fibronectin (Woods et al., 1986) was a generous gift from Dr. John Couchman (University of Alabama at Birmingham, Birmingham, AL) and was used at 0.05 mg/ml; laminin (ICN Biomedicals, Inc., Costa Mesa, CA) was used at 0.1 mg/ml; vitronectin (Sigma Chemical Co.) was used at 0.025 mg/ml; Pronectin-F (Protein Polymer Technologies, Inc., San Diego, CA) was used at 0.1 mg/ml. For some experiments, rat embryo fibroblasts were treated with various concentrations of herbimycin A (37°C), BRL, Gaithersburg, MD) dissolved in DMSO. Protein was precipitated with 2 M urea, 10% trichloroacetic acid or 5% trichloroacetic acid. The precipitate was washed once in cold TBS and then dissolved in 1 N NaOH, and protein was determined spectrophotometrically. Protein was then resuspended in TBS before addition to the blots. All blots were equilibrated with TBS containing 0.1% Triton X-100 before addition of the antibody. The antibody was incubated with the blots for 2 h at room temperature before washing and development with chemiluminescent reagents. The autoradiographs were exposed for \( 10^3-10^5 \) min.

**SDS-PAGE and Western Blot Analysis**

For SDS gel electrophoresis dishes were drained of medium and the cells were dissolved by scraping with a Teflon policeman in 200 \( \mu \)l of Laemmli sample buffer containing 1 mM Na orthovanadate. The samples were boiled for 2 min and the DNA was sheared by passing several times through a 26-gauge needle. Samples containing equal protein concentrations were electrophoresed on 7.5% or 10% polyacrylamide gels (Laemmli, 1970) with bisacrylamide concentrations of 0.19 and 0.13%, respectively. After electrophoresis, gels were either stained with Coomassie blue or transferred to nitrocellulose (Towbin et al., 1979). After blocking of the nitrocellulose, phosphoryrosine-containing proteins were visualized in most cases by incubation with 125I-labeled p20 (\( \sim 2 \times 10^4 \) cpm/ml) for 60 min. After washing for 60 min, the nitrocellulose was exposed with an intensifying screen to x-ray film for autoradiography. P20 was the generous gift of Dr. John Gleave (University of Kentucky, Lexington, KY) or was purchased from ICN Biomedicals, Inc. P20 was radioiodinated using iodogen (Pierce Chemical Co., Rockford, IL) (Frazier and Speck, 1978). Iodine-125 was purchased from New England Nuclear (Boston, MA). In some experiments, phosphoprotein-containing proteins were visualized by incubating with a 1:1,500 dilution of the antibody PT66 (Sigma Chemical Co.), followed by affinity-purified rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) followed by 125I-protein A at 8 \( \times 10^5 \) cpm/ml (a gift from Dr. S. Earp, University of North Carolina at Chapel Hill, NC).

**Immunoprecipitation**

Cells were lysed by scraping in 150 mM NaCl, 0.1% Na dodecyl sulfate (SDS), pH 7.6 (TBS) that contained 2 mM EDTA, 1 mM Na orthovanadate, 10 \( \mu \)g/ml leupeptin, 0.1% Triton X-100, and 0.1% Na deoxycholate. The lysates were clarified by centrifugation at 100,000 g for 30 min. Anti-phosphotyrosine antibody coupled directly to agarose beads was added to the supernatants and incubated with end-over-end mixing for 90 min at 4°C. The beads were sedimented by brief centrifugation at 12,000 g and washed extensively with lysis buffer. Phosphotyrosine-containing proteins were released from the antibody by incubating on ice for 30 min in 10 mM phenylphosphate in lysis buffer containing 0.1 mg/ml ovomucin as carrier protein. The beads were pelleted and the supernatant was used to precipitate paxillin in a similar manner by incubating with agarose beads coupled with anti-paxillin (antibody 165) (Glezen and Zolot, 1989; Turner et al., 1990). Other lysates were incubated with monoclonal anti-pp125FABK (antibody 2A7; generous gift of Tom Parsons, University of Virginia, Charlottesville, VA; Schaller et al., 1992). Rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) bound to protein A Sepharose was then added to these samples. Beads from all of the above experiments were sedimented by brief centrifugation after immunoprecipitation and washed extensively with lysis buffer. Proteins were then released for SDS-PAGE and blot analysis by boiling in Laemmli sample buffer with 1 mM Na orthovanadate for 3 min.

**Fluorescence Microscopy**

For immunofluorescence microscopy cells were fixed in 3.7% formaldehyde in Dulbecco's phosphate-buffered saline for 8 min, rinsed in TBS, and permeabilized for 5 min in TBS containing 0.5% Triton X-100. The coverslips were then incubated at 37°C in p20 (10 \( \mu \)g/ml) mixed with affinity-purified rabbit anti-human talin. (In some experiments the coverslips were stained with p20 before staining with the anti-talin antibody, but no significant differences were detected in the resulting staining patterns). After 45 min the coverslips were rinsed extensively in TBS and then stained with a mixture of rhodamine-labeled, affinity-purified goat anti–mouse IgG (Chemicon International Inc., Temecula, CA) and fluorescein-labeled, affinity-purified goat anti–rabbit IgG (Chemicon International Inc.) for 45 min at 37°C. Other coverslips were incubated with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR), mixed with anti-paxillin antibody, and then stained with affinity-purified goat anti–mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) bound to protein A Sepharose and then stained with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR).
Results

Distribution of Phosphotyrosine in Cultured Fibroblasts

The distribution of phosphotyrosine-containing proteins in rat embryo fibroblasts (REF52) plated in serum-free medium for different intervals on a fibronectin-coated surface was examined by immunofluorescence microscopy using the monoclonal antibody py20 (Fig. 1). After 1 h of adhesion the most prominent localization of phosphotyrosine-containing proteins was in the focal adhesions, revealed by double-labeling with antibodies against talin. This result was consistent with previous observations (Maher et al., 1985). At early time points after plating (e.g., 10-15 min) no focal adhesions could be detected and the cells were well rounded (data not shown). As the cells spread, focal adhesions became increasingly prominent and in many cells the staining patterns with anti-phosphotyrosine and anti-talin appeared to be closely coincident in terms of focal adhesion staining. However, with the anti-phosphotyrosine antibody, the staining of adhesions at the cell periphery was often brighter than the staining of more central adhesions. This was particularly true where cells came into contact with each other and in cells plated for long periods (Fig. 1, C-F). In some cases the more central adhesions stained only very weakly, if at all, for phosphotyrosine, but appeared to be uniformly stained with antibodies against talin (Fig. 1, C-F). To ensure that this staining pattern was not the result of the polyclonal anti-talin competing with or blocking the monoclonal anti-phosphotyrosine, these antibodies were also used sequentially with the monoclonal being used first. This did not affect the observed patterns (data not shown). Since focal adhesions tend to form at the periphery (Izzard and Lochner, 1980; DePasquale and Izzard, 1987) where the anti-phosphotyrosine staining is often brightest, we suspect that the tyrosine phosphorylation of focal adhesion proteins is associated more with the formation of these structures than with their maintenance or disassembly.

Western Blot Analysis of Tyrosine Phosphorylation in Response to ECM Adhesion

To examine which proteins contain phosphotyrosine, extracts of REF52 plated on fibronectin in serum-free medium for different periods were electrophoresed on SDS gels, transferred to nitrocellulose, and blotted with 125I-py20 (Fig. 2). A relatively simple profile of phosphotyrosine-containing bands was revealed and a subset of these proteins showed a marked increase in phosphotyrosine content with increasing time of adhesion to fibronectin as compared with cells kept in suspension. These had approximate molecular masses of 130, 115, and 70 kD. It should be noted that when cells were kept in suspension the level of tyrosine phosphorylation decreased progressively until it was essentially undetectable by ~90 min (data not shown). However, when these suspended cells were plated on fibronectin they developed the same pattern of bands showing elevated phosphotyrosine with the same time course (data not shown).

We were interested in whether this elevation in tyrosine phosphorylation could be induced by adhesion to other substrates. REF52 plated on uncoated plastic or on polylysine failed to show an elevation in phosphotyrosine (data not shown), consistent with the results of Guan et al. (1991). To investigate whether adhesion to ECM proteins other than fibronectin would promote tyrosine phosphorylation, REF52 were plated in serum-free medium on dishes that had been coated with fibronectin, vitronectin, or laminin. REF52 were observed to adhere well and spread on each of these proteins. The cells were harvested in sample buffer 1 h after plating and analyzed by Western blotting with 125I-py20 (Fig. 3). Compared with adhesion to uncoated plastic, in each case the adhesion of REF52 to fibronectin, vitronectin, and laminin generated the same pattern of bands that were elevated in phosphorytrosine. To check that this effect was not due to the cells interacting with their own secreted fibronectin, parallel cultures were stained with anti-fibronectin and examined by immunofluorescence microscopy. No extracellular fibronectin was detected (data not shown).

Since fibronectin, vitronectin, and laminin share the characteristic that they all interact with cells via members of the integrin family of ECM receptors, the elevated phosphotyrosine induced by adhesion to these proteins is consistent with the earlier work implicating integrins in tyrosine phosphorylation (Guan et al., 1991; Kornberg et al., 1991). To further explore the role of integrins in this response, REF52 were plated on the cell-binding fragment of fibronectin. This contains the RGD sequence that is involved in binding to the major fibronectin receptor, the integrin $\alpha_5\beta_1$. RGD sequences are present in many other ECM proteins, including laminin and vitronectin, and contribute to the binding of appropriate integrins to these proteins. REF52 were also plated on Pronectin-F, a synthetic polymer that contains multiple RGD sequences and that is presumably recognized by one or more integrins (Fig. 3). The REF52 spread well on the cell-binding fragment of fibronectin, but spread less effectively on Pronectin-F. Analysis by Western blotting revealed that, compared with adhesion to uncoated plastic, the cells on the cell-binding fragment of fibronectin and on Pronectin-F both contained elevated phosphotyrosine in the 70-, 115-, and 130-kD bands. The elevation was less on Pronectin-F, correlating with the less extensive adhesion and spreading observed on this substrate.

We have examined the pattern of tyrosine phosphorylation in several cell types in response to adhesion to fibronectin. Elevated phosphotyrosine in proteins between 115 and 130 kD were observed in human umbilical vein endothelial cells, chicken embryo fibroblasts, BHK cells (data not shown), NIH 3T3 cells, and Balb/c 3T3 mouse fibroblasts. We noted, however, that NIH and Balb/c 3T3 cells were able to spread to some extent on uncoated plastic in the absence of serum and demonstrated an elevation in phosphotyrosine during this adhesion and spreading. It seemed likely that this was due to secretion by the cells of some ECM component that adsorbed to the plastic and promoted cell spreading. To prevent synthesis and secretion of ECM components by these cells, they were pretreated for 2 h with cycloheximide and plated in its presence. Under these conditions, cell spreading on surfaces coated with fibronectin was unaffected but cell spreading on plastic was greatly reduced. Analysis of the pattern of phosphotyrosine-containing proteins for Balb/c and NIH 3T3 cells is shown in Fig. 4. Compared with sus-
Localization of phosphotyrosine in REF52 plated on fibronectin. Cells were plated on fibronectin-coated coverslips in serum-free media for 75 min (A and B) or 20 h (C-F). The cells were stained for phosphotyrosine (A, C, and E) and talin (B, D, and F) to reveal the focal adhesions. Note that the most prominent phosphotyrosine localization is in the focal adhesions. However, the phosphotyrosine is often more prominent in the peripheral adhesions relative to the more central ones. In some of these the phosphotyrosine staining is less extensive (arrows in C and E) than the staining for talin. Bar = 20 μm.

pension conditions, both cell types revealed an elevation in phosphotyrosine in a doublet of bands in the 115-130-kD range in response to adhesion to fibronectin. On plastic a low level of tyrosine phosphorylation was detected. Similar patterns of tyrosine phosphorylation were obtained in Western blots performed with two different anti-phosphotyrosine monoclonal antibodies, py20 and PT66.

Identification of the Phosphotyrosine-containing Proteins

To investigate the identity of the proteins that become tyrosine-phosphorylated in response to adhering to ECM components, we have used immunoprecipitation and Western blotting. REF52 cells were either plated on a fibronectin-
Changes in protein phosphotyrosine levels during cell spreading on fibronectin. REF52 were kept in suspension (lane 1) or plated on fibronectin for 15 min (lane 2), 30 min (lane 3), or 3 h (lane 4). Cell lysates were electrophoresed on polyacrylamide gels and either stained with Coomassie blue (right) or transferred to nitrocellulose and blotted with \( ^{125}\text{I}-\text{py20} \) to reveal phosphotyrosine-containing proteins (left). \( M \), marker proteins (radiolabeled in the left panel). The molecular masses of marker proteins are indicated in kilodaltons to the left of the autoradiograph. Increased phosphotyrosine was detected in bands at \(~130, 115, \text{and} 70 \text{kD} \) with increasing times of adhesion on fibronectin.

It occurred to us that the 70-kD phosphoprotein in the REF52 might be paxillin, a focal adhesion protein that is a known substrate for the tyrosine kinase, pp60\(^{src} \) (Glennay and Zokas, 1989), as well as a phosphotyrosine-containing protein in normal cells during development (Turner, 1991). To test whether this 70-kD protein might be paxillin, REF52 were kept in suspension or plated on fibronectin-coated dishes for 0.5, 1, or 8 h. The cells were lysed and the phosphotyrosine-containing proteins immunoprecipitated with \( ^{125}\text{I}-\text{py20} \). These phosphotyrosine-containing proteins were released from the py20 with phenylphosphate and then immunoprecipitated with anti-paxillin. These immunoprecipitates were run on an SDS gel, transferred to nitrocellulose, and blotted with py20 (Fig. 6). The cells in suspension contained very little paxillin that was phosphorylated on tyrosine, but the level of phosphotyrosine in paxillin increased markedly over the time course of cell adhesion to fibronectin, thus confirming the identity of the 70-kD band as paxillin.

To quantify the level of tyrosine phosphorylation on paxillin, REF52 were metabolically labeled with \([^{38}\text{S}]\text{methionine}\) for 36 h. The cells were kept in suspension for 1 h to reduce phosphotyrosine levels to a minimum and then plated on fibronectin for 1 h before lysis. The lysate was divided into two: one half was immunoprecipitated with anti-paxillin and the other half was immunoprecipitated with the anti- phosphotyrosine antibody, py20. The immunoprecipitations were repeated three times to insure that all of the paxillin and all of the phosphotyrosine-containing proteins were precipitated. The immunoprecipitates were electrophoresed on an SDS gel. After drying the gel and autoradiography, the bands corresponding to paxillin were cut out and the radioactivity was determined by scintillation counting. Counts were 1,004 and 4,344 cpm from the py20 and paxillin immunoprecipitations, respectively. Thus, comparison of the amounts of paxillin precipitated with py20 and with anti-paxillin indicated that 23% of the paxillin had become phosphorylated on tyrosine in response to cell adhesion to fibronectin for 1 h.

A phosphotyrosine-containing band in the region of paxillin is less obvious in the Balb/c 3T3 cells plated on fibronectin than in the NIH 3T3 cells or in the REF52 cells (Fig. 4). This could be because the level of paxillin is lower in the Balb/c 3T3 cells. To analyze directly whether there is a change in the level of tyrosine phosphorylation of paxillin in response to adhesion in these cells, Balb/c 3T3 cells were either kept in suspension or plated on fibronectin. After 1 h the cells were lysed and immunoprecipitated with py20. The immunoprecipitates were released with phenylphosphate.
Figure 4. Changes in phosphotyrosine levels in Balb/c 3T3 and NIH 3T3 cells spreading on fibronectin. Balb/c 3T3 cells (A and B) or NIH 3T3 cells (C and D) were kept in suspension (lane 1), plated on plastic for 20 min (lane 2) and 60 min (lane 3), or plated on fibronectin for 20 min (lane 4) and 60 min (lane 5). B and D show gels stained with Coomassie blue. A and C show corresponding autoradiographs of Western blots probed with the anti-phosphotyrosine monoclonal antibody PT66, followed by rabbit anti-mouse IgG, followed by 125I-protein A. Note the prominent increase in the phosphotyrosine-containing proteins between 115 and 130 kD in response to adhesion to fibronectin. M, marker proteins (radiolabeled in A and C). The molecular masses of marker proteins are indicated in kilodaltons to the left of A.

Inhibition of Tyrosine Phosphorylation Inhibits Focal Adhesion Formation

We have used tyrosine kinase inhibitors to analyze the function of the tyrosine phosphorylation induced by cell adhesion to fibronectin. The tyrosine kinase inhibitor herbimycin A was used at a concentration of 875 nM after reconstitution in DMSO. At this concentration we have found no toxic...
effects on human vascular endothelial cell cultures maintained for 96 h, although cell division is inhibited (Romero, L. H., unpublished observations). (Others have demonstrated that 5 μM herbimycin A may be used in experiments with rat vascular smooth muscle cells without any evidence of cytotoxicity [Weiss and Nuccitelli, 1992]). In these experi-
ments, pretreatment of REF52 with 875 nM herbimycin A and inclusion of this agent in the medium during adhesion to fibronectin resulted in a marked decrease in tyrosine phosphorylation (Fig. 8). The major bands exhibiting phosphorylation on tyrosine in whole cell extracts of REF52 reveal only trace amounts of tyrosine phosphorylation in extracts of herbimycin A-treated REF52 plated on fibronectin for 1 h. Using 220 nM herbimycin A resulted in a decreased level of tyrosine phosphorylation that was intermediate between the levels for untreated cells and cells in 875 nM herbimycin A (data not shown). Cells treated with 55 nM herbimycin A and DMSO-treated cells showed no difference from control conditions (data not shown). The specific effect of 875 nM herbimycin A on the phosphorylation of pp125FAK is shown in Fig. 5 (lane 3). The level of phosphorylation of pp125FAK is comparable to that in cells kept in suspension without plating.

REF52 spread on fibronectin in the presence of 875 nM herbimycin A, although spreading was less extensive than that seen in untreated controls. The formation and organization of focal adhesions in the presence and absence of herbimycin A was analyzed by immunofluorescence microscopy using antibodies against phosphotyrosine, talin, and paxillin (Fig. 9). Optimal comparison of focal adhesion staining between herbimycin A-treated cells and untreated cells was achieved by selecting only treated cells with the most normal morphology and spreading for inclusion in these figures. Consistent with the Western blot analysis indicating decreased tyrosine phosphorylation in the presence of 875 nM herbimycin A, anti-phosphotyrosine antibodies revealed significantly decreased staining in treated cells. With py20, many of the cells treated with herbimycin A showed little focal adhesion staining. The staining that remained was most prominent in the perinuclear region and at the cell periphery (Fig. 9, A and B). Double-labeling with anti-talin antibody was used in these same cells to determine whether the diminished phosphotyrosine staining seen in focal adhesions with herbimycin A treatment was associated with decreased focal adhesion assembly (Fig. 9, C and D). Many herbimycin A-treated cells showed a marked decrease in focal adhesions as judged by talin staining. Anti-paxillin antibody staining corroborated this paucity or complete loss of focal adhesions in many of the cells treated with herbimycin A (Fig. 9, E and F). Actin cytoskeletal organization in response to cell adhesion was also disrupted in these cells as evidenced by sparse actin filaments (stress fibers) in cells stained with rhodamine-conjugated phalloidin (Fig. 9, G and H). The effects of lower concentrations of herbimycin A were also explored. REF52 pretreated with and plated in the presence of 220 nM herbimycin A also showed decreased formation of focal adhesions and stress fibers compared with untreated cells (data not shown). However, the inhibition was less pronounced than that obtained with 875 nM herbimycin A. Cells treated with 55 nM herbimycin A or with DMSO alone showed no detectable changes in the patterns of phosphotyrosine, talin, paxillin, and actin as compared with untreated controls (data not shown).

The reversibility of the herbimycin A effects was also investigated. After pretreatment with 875 nM herbimycin A for 24 h and plating for 1 h on fibronectin in the presence of herbimycin A, coverslips were rinsed and returned to normal medium without serum. Within 4 h there was a restora-
Figure 9. Inhibition of focal adhesion and stress fiber formation by herbimycin A. REF52 were plated on fibronectin-coated coverslips for 1 h in the absence (A, C, E, and G) or presence of 875 nM herbimycin A (B, D, F, and H). (Cells plated in the presence of herbimycin A had been pretreated with 875 nM for 24 h.) Cells were stained for phosphotyrosine (A and B), talin (C and D), paxillin (E and F), or actin (G and H). A double-labeling technique was used to document the colocalization of phosphotyrosine and talin in focal adhesions (A and C, and B and D, respectively), and the concomitant changes in paxillin in focal adhesions with those in stress fibers (E and G, and F and H, respectively). Scale bar = 30 μM.
tion of the normal pattern of focal adhesions and stress fibers, whereas parallel cultures maintained in herbimycin A continued to show inhibition of focal adhesion and stress fiber formation (data not shown). The effects of herbimycin A on fully spread cells was also examined. REF52 were treated with 875 nM herbimycin A 24 h after plating. After both 24 and 48 h of herbimycin A treatment, these cells continued to show prominent stress fibers and focal adhesions, but showed reduced levels of phosphotyrosine in their focal adhesions (data not shown).

**Discussion**

It has been shown recently that clustering of integrins on the surface of cells or binding of integrins to ECM ligands stimulates tyrosine phosphorylation of a number of proteins that are distinct from the integrin α and β chains themselves (Guan et al., 1991; Kornberg et al., 1991). In this paper we have identified two of these proteins that become tyrosine-phosphorylated in response to cell adhesion to ECM ligands. One of these proteins, pp125FAK, is a novel tyrosine kinase that is concentrated in focal adhesions (Schaller et al., 1992). It is possible that this kinase is responsible for the increased tyrosine phosphorylation induced by adhesion and detected in the focal adhesions of spread cells. However, a direct association between pp125FAK and integrins has yet to be demonstrated. Additionally, pp125FAK may not be the primary kinase, but instead a substrate for some other tyrosine kinase. In this respect, it should be noted that in cells transformed by Rous sarcoma virus, pp125FAK is a substrate for the tyrosine kinase pp60src (Kanner et al., 1990), which is also concentrated in focal adhesions.

The second protein with elevated phosphotyrosine in cells adhering to fibronectin that we have identified is paxillin. Paxillin is a vinculin-binding protein concentrated in focal adhesions (Turner et al., 1990). Paxillin is also one of the major tyrosine kinase substrates in developing tissues (Turner, 1991). This finding, together with the absence of tyrosine phosphorylation on paxillin in adult tissues, is consistent with our current observations that suggest that the tyrosine phosphorylation of paxillin is associated with the formation of cytoskeleton–membrane attachments, rather than with the stabilization and maintenance of these structures. One possible role for the tyrosine phosphorylation of paxillin is the generation of a binding site that is recognized by a src homology 2 domain in another protein. src homology 2 domains have been identified in several proteins involved in signal transduction and have been shown to bind to phosphotyrosine residues within specific sequences of other proteins (Koch et al., 1991). Tyrosine phosphorylation regulates this interaction. Recently, a src homology 2 domain has been identified within tensin, an actin-binding, focal adhesion protein (Davis et al., 1991). It will be important to determine whether there is an interaction between paxillin and tensin, or another focal adhesion protein, that is regulated by this tyrosine phosphorylation of paxillin. Such an interaction could be instrumental in triggering the assembly of the actin cytoskeleton as focal adhesions develop.

The tyrosine phosphorylation of paxillin has also been detected in cells transformed by Rous sarcoma virus (Glenny and Zokas, 1989). Since transformation of cells by Rous sarcoma virus is associated with disruption of focal adhesions, elevated phosphotyrosine in focal adhesion proteins has usually been thought of in terms of focal adhesion disassembly. However, we present here three lines of evidence indicating that tyrosine phosphorylation is involved in cytoskeletal assembly rather than disassembly in normal cells spreading on an ECM substrate. First, the increase in phosphotyrosine is induced by adhesion and spreading and not by detachment of cells from the ECM. Second, there is the observation that in some cells the more central and mature focal adhesions stain less strongly for phosphotyrosine than the focal adhesions at the cell periphery (Fig. 1). Typically, focal adhesions form at the cell periphery (Izzard and Lochner, 1980; Depasquale and Izzard, 1987), indicating that it is the newly formed adhesions that contain elevated phosphotyrosine. Third, treatment of cells with the tyrosine kinase inhibitor herbimycin A interfered with the increase in tyrosine phosphorylation in response to cell adhesion to fibronectin and also inhibited the formation of focal adhesions and their associated stress fibers in many cells. It should be noted that cells that had already spread and developed focal adhesions and stress fibers before herbimycin A treatment continued to show these structures after the addition of herbimycin A. This further suggests that tyrosine phosphorylation is involved in the initial formation of these structures and not in their maintenance.

Although our evidence indicates a role for tyrosine phosphorylation in the formation of focal adhesions, tyrosine phosphorylation of these proteins is not sufficient for focal adhesions to develop. Cells plated on the cell-binding fragment of fibronectin adhere, spread, and reveal elevated phosphotyrosine in the same proteins as cells plated on intact fibronectin (Fig. 3). However, on the cell-binding fragment of fibronectin, cells fail to develop normal focal adhesions (Izzard et al., 1986; Woods et al., 1986; our unpublished observations). In addition to the cell-binding fragment of fibronectin, a heparin-binding fragment is also required for focal adhesions to develop. Woods and Couchman have presented evidence that the heparin-binding fragment of fibronectin exerts its effect by activating protein kinase C (Woods and Couchman, 1992). It appears, therefore, that at least two signals are required for the formation of focal adhesions, one involving protein kinase C and the other involving tyrosine kinase activation.

Tyrosine kinase inhibitors may be useful in defining a direct or indirect role for specific kinases in the adhesion-induced tyrosine phosphorylation of focal adhesion proteins. In this paper we have concentrated on the tyrosine kinase inhibitor herbimycin A, a widely effective inhibitor of tyrosine kinase activity (Uehara et al., 1989; Fukazawa et al., 1990; June et al., 1990; Iwasaki et al., 1992; Otani et al., 1992; Weiss and Nuccitelli, 1992). However, in preliminary work we have also observed inhibition of focal adhesion formation in REF52 by various tyrphostins (Romer, L. H., unpublished results). Whereas the tyrphostins are competitive inhibitors of tyrosine kinases, herbimycin A inhibits pp60src by binding of reactive sulfhydryl groups on pp60src to the benzquinone moiety of the inhibitor and blocking the availability of an active site (Uehara et al., 1989; Fukazawa et al., 1990). The effects of herbimycin A that we report here include the inhibition of adhesion-induced tyrosine phosphorylation of focal adhesion proteins including pp125FAK and paxillin; the inhibition of focal adhesion formation; and
interference with stress fiber assembly in normal cells plated on fibronectin. It will be important to determine whether herbinycin A has a direct effect on pp125FAK activity, or whether it is affecting some other kinase that is active earlier in the signaling cascade and is directly triggered by integrin clustering or occupancy. The use of a panel of tyrosine kinase inhibitors with different specificities may be useful in delineating the cascade of events leading to the phosphorylation of pp125FAK and paxillin, and to the formation of focal adhesions and stress fibers in response to adhesion to ECM.

Herbinycin A and other more specific tyrosine kinase inhibitors may also be instrumental in determining the role of tyrosine phosphorylation in other adhesion-induced events, such as anchorage-dependent growth behavior. The basis for this requirement for adhesion to a substrate in order for normal cells to respond to growth factors by DNA replication and cell division has been an enigma, although various possible explanations have been advanced (see Burridge, 1986, for a review). Anchorage dependence of growth has recently been correlated with the level of intracellular pH (Ingber et al., 1990; Schwartz et al., 1989, 1990a,b). An alternative or hypothetical possibility is that adhesion-induced phosphotyrosine-containing proteins may serve a role in growth control as well as in cytoskeletal organization.

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The Journal of Cell Biology, Volume 119, 1992 902

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