Tyrosine Kinase Activity, Cytoskeletal Organization, and Motility in Human Vascular Endothelial Cells

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Tyrosine phosphorylation of cytoskeletal proteins occurs during integrin-mediated cell adhesion to extracellular matrix proteins. We have investigated the role of tyrosine phosphorylation in the migration and initial spreading of human umbilical vein endothelial cells (HUVEC). Elevated phosphotyrosine concentrations were noted in the focal adhesions of HUVEC migrating into wounds. Anti-phosphotyrosine Western blots of extracts of wounded HUVEC monolayers demonstrated increased phosphorylation at 120–130 kDa when compared with extracts of intact monolayers. The pp125^{FAK} immunoprecipitated from wounded monolayers exhibited increased kinase activity as compared to pp125^{FAK} from intact monolayers. The time to wound closure in HUVEC monolayers was doubled by tyrphostin AG 213 treatment. The same concentration of AG 213 interfered with HUVEC focal adhesion and stress fiber formation. AG 213 inhibited adhesion-associated tyrosine phosphorylation of pp125^{FAK} in HUVEC. Tyrphostins AG 213 and AG 808 inhibited pp125^{FAK} activity in in vitro kinase assays. pp125^{FAK} immunoprecipitates from HUVEC treated with both of these inhibitors also had kinase activity in vitro that was below levels seen in untreated HUVEC. These findings suggest that tyrosine phosphorylation of cytoskeletal proteins may be important in HUVEC spreading and migration and that pp125^{FAK} may mediate phosphotyrosine formation during these processes.

INTRODUCTION

The extracellular matrix influences many aspects of cell behavior. Recently, much attention has focused on the signaling pathways that are associated with integrin binding to extracellular matrix ligands (Burridge et al., 1992a; Hynes, 1992; Juliano and Haskill, 1993). An elevation in tyrosine phosphorylation has been observed in response to both integrin clustering (Kornberg et al., 1991) and integrin-mediated cell adhesion (Guan et al., 1991; Burridge et al., 1992b). One of the proteins that is phosphorylated on tyrosine during integrin-mediated cell adhesion is pp125^{FAK} (Burridge et al., 1992b; Guan and Shalloway, 1992; Hanks et al., 1992; Kornberg et al., 1992; Lipfert et al., 1992). pp125^{FAK} is a tyrosine kinase that has been localized to focal adhesions in fibroblasts (Hanks et al., 1992; Schaller et al., 1992) and that may serve an important role in signal transduction during cell interactions with extracellular matrix, neu-

ropeptides, and oncogenes (Zachary et al., 1992; Zachary and Rozengurt, 1992). The adhesion-associated increase in pp125^{FAK} tyrosine phosphorylation has been shown to correlate with increased tyrosine kinase activity (Guan and Shalloway, 1992; Lipfert et al., 1992).

In vascular endothelium, interactions with extracellular matrix result in changes in cell morphology, growth, and motility (Young and Herman, 1985; Kubota et al., 1988; Madri et al., 1988; Ingber and Folkman, 1989; Yost and Herman, 1990). The integrin family of adhesion receptors is directly involved in endothelial cell binding to a variety of extracellular matrix proteins and may modulate phenotypic changes associated with inflammation (Albelda et al., 1989; Albelda and Buck, 1990; Defilippi et al., 1992). The complex sheet migration response of endothelial cells to mechanical wounding in vitro involves cell spreading and migration on an extracellular matrix, as well as cell proliferation (Coom-

ber and Gotlieb, 1990). Cytoskeletal mechanisms have been implicated in regulating these processes (Gotlieb et al., 1981; Wong and Gotlieb, 1984). We have investigated tyrosine phosphorylation events during the endothelial cell wound healing response in an attempt to elucidate signal transduction mechanisms that mediate cell-extracellular matrix interactions.

We have found increased tyrosine phosphorylation in the focal adhesions of migrating human umbilical vein endothelial cells (HUVEC) adjacent to wounds. pp125^{FAK} is one of the proteins demonstrating increased tyrosine phosphorylation in wounded HUVEC monolayers and is concentrated in focal adhesions in these cells. Tyrosine kinase inhibitor treatment of HUVEC retards wound healing and inhibits cytoskeletal organization. The same inhibitors also affect pp125^{FAK} tyrosine phosphorylation and kinase activity. These findings imply a role for pp125^{FAK}-mediated tyrosine phosphorylation in focal adhesion assembly and in the endothelial cell response to mechanical injury.

MATERIALS AND METHODS

Cell Culture, Monolayer Wounding, and Protein Tyrosine Kinase Inhibitors

HUVEC were harvested from fresh human umbilical veins using modifications of previously described techniques (Gimbrone et al., 1974). Segments of vessels were cannulated, gently irrigated to remove debris, and incubated with 1 mg/ml collagenase (Worthington Biochemical, Freehold, NJ) for 10 min. The irrigant was then agitated within the vessels and collected. Harvested cells were plated on tissue culture plastic that had been preincubated with 1% gelatin (Difco, Detroit, MI) in Dulbecco's phosphate-buffered saline (PBS). HUVEC were cultured in medium 199 with Earle's salts, supplemented with vitamins and amino acids (GIBCO BRL, Gaithersburg, MD), 20% fetal calf serum, 100 μg/ml heparin, ~100 μg/ml endothelial cell growth factor isolated from bovine hypothalamus, 50 μ/ml penicillin, 50 μg/ml streptomycin, and 4 μg/ml amphotericin B. HUVEC were used between passages two and four. Cells were passaged 7 d before use. Verification of HUVEC identity was accomplished by morphology and by positive testing for von Willebrand's factor by immunofluorescence microscopy with polyclonal antibody obtained from Sigma Chemical (St. Louis, MO).

For wounding experiments and gel and blot analysis, cells were plated at ~6 × 10^4 cells/cm^2. Coverslips or culture dishes were coated with human plasma fibronectin (New York Blood Center, NY) (75 μg/ml) by incubation at 37°C for 60 min or in some experiments by incubation overnight at 4°C. Before plating cells, coverslips or dishes were rinsed twice in serum-free Dulbecco's modified Eagle's medium. Single wounds were made in confluent monolayers by dragging a sterile 1-mm pipette tip across the monolayer to create a cell-free path. Multiple wounds were made by using a comb-like device fashioned from soft nylon bristles cemented to a plastic frame. This device was sterilized for each use. For multiple wounding experiments, monolayers were cultured in the absence of endothelial cell growth factor and heparin for 72 h before wounding and were allowed to recover for 96 h after wounding, before harvest for blot and immunoprecipitation analysis. These monolayers were not supplied with fresh serum for 7 d before harvesting. For adhesion experiments, cells were trypsinized, washed once in medium containing 10% serum, washed once in serum-free medium, resuspended, and plated in serum-free medium.

For some experiments, HUVEC were treated with various concentrations of tyrphostins AG 213 or AG 808 (generous gifts of Alexander Levitski, The Hebrew University of Jerusalem) or of herbimycin A (GIBCO BRL). Inhibitors were dissolved in dimethyl sulfoxide (DMSO) before use in tissue culture medium. Cells were pretreated with inhibitors for 24 h before plating on fibronectin in the presence of the same inhibitor at the same concentration. Confluent HUVEC monolayers were also pretreated with inhibitors for 24 h before wounding. Recovery from tyrphostin effects was studied in monolayers incubated in medium free of inhibitors for various times.

Fluorescence Microscopy

Cells were fixed in 3.7% formaldehyde in Dulbecco's PBS for 10 min, rinsed in 150 mM NaCl, 0.1% NaN3, 50 mM tris(hydroxymethyl)-aminomethane HCl pH 7.6 (TBS) and permeabilized for 5 min in TBS containing 0.5% Triton X-100 before staining for immunofluorescence microscopy. The coverslips were blocked with 4% horse serum (GIBCO BRL) in TBS for 30 min at 37°C before incubations with antibodies. Coverslips were then incubated for 30 min with py20 (ICN Biochemicals, Costa Mesa, CA) in TBS with 4% horse serum. The coverslips were then rinsed extensively in TBS and then stained with fluorescein-conjugated, affinity-purified goat anti-mouse IgG (Jackson Immuno-Research, West Grove, PA) for 30 min at 37°C. For visualization of pp125^{FAK}, coverslips were incubated with antibody 2A7 (generous gift of Drs. J. Thomas Parsons and Michael Schaller, University of Virginia) (Schaller et al., 1992) and then stained with a second antibody as described above. Other coverslips were incubated with py20 mixed with affinity-purified rabbit anti-human talin and then stained with a mixture of rhodamine-labeled, affinity-purified goat anti-mouse IgG (Chemicon International, Temecula, CA) and fluorescein-labeled, affinity-purified goat anti-rabbit IgG (Chemicon International) for 30 min at 37°C. For visualization of filamentous actin, coverslips were incubated with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for 30 min at 37°C. After the antibody incubations, the coverslips were washed in TBS, rinsed in deionized water, and mounted with gelvatol or mowiol. Coverslips were viewed on a Zeiss Axiophot microscope (Thornwood, NY) equipped for epifluorescence. Fluorescence micrographs were taken on T-max 400 film (Eastman Kodak, Rochester, NY).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

After several washes with serum-free medium and PBS, cells were prepared for SDS gel electrophoresis of whole cell lysates by scraping with a teflon policeman in 100 μl of Laemmli sample buffer containing 1 mM Na orthovanadate. The samples were boiled for 3 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% SDS-polyacrylamide gels (Laemmli, 1970) with a bisacrylamide concentration of 0.19%. After electrophoresis, gels were either stained with Coomassie blue or transferred to nitrocellulose (Towbin et al., 1979). After blocking of the nitrocellulose, phospho-tyrosine-containing proteins were visualized in some experiments by incubation with ^{125}I-labeled py20 (~2 × 10^6 cpm/ml, ICN Biochemicals) for 60 min. After washing for 60 min, the nitrocellulose was exposed with an intensifying screen to x-ray film for autoradiography. Iodine-125 was purchased from New England Nuclear (Wilmington, DE), and py20 was radio-iodinated using iodogen (Pierce Chemical, Rockford, IL) according to previously described protocols (Fraker and Speck, 1978). Immunoprecipitates were subjected to blot analysis using enhanced chemiluminescence (Amersham, Arlington Heights, IL) with horseradish peroxidase (HRP)-conjugated py20 (ICN Biochemicals) and techniques described previously (Bockholt et al., 1992). Nitrocellulose membranes that were analyzed by this technique were incubated for 30 min with blocking buffer (TBS with 2.5% bovine serum albumin [BSA] and 0.05% Tween 20) before the antibody incubation.

wound edge   intact monolayer
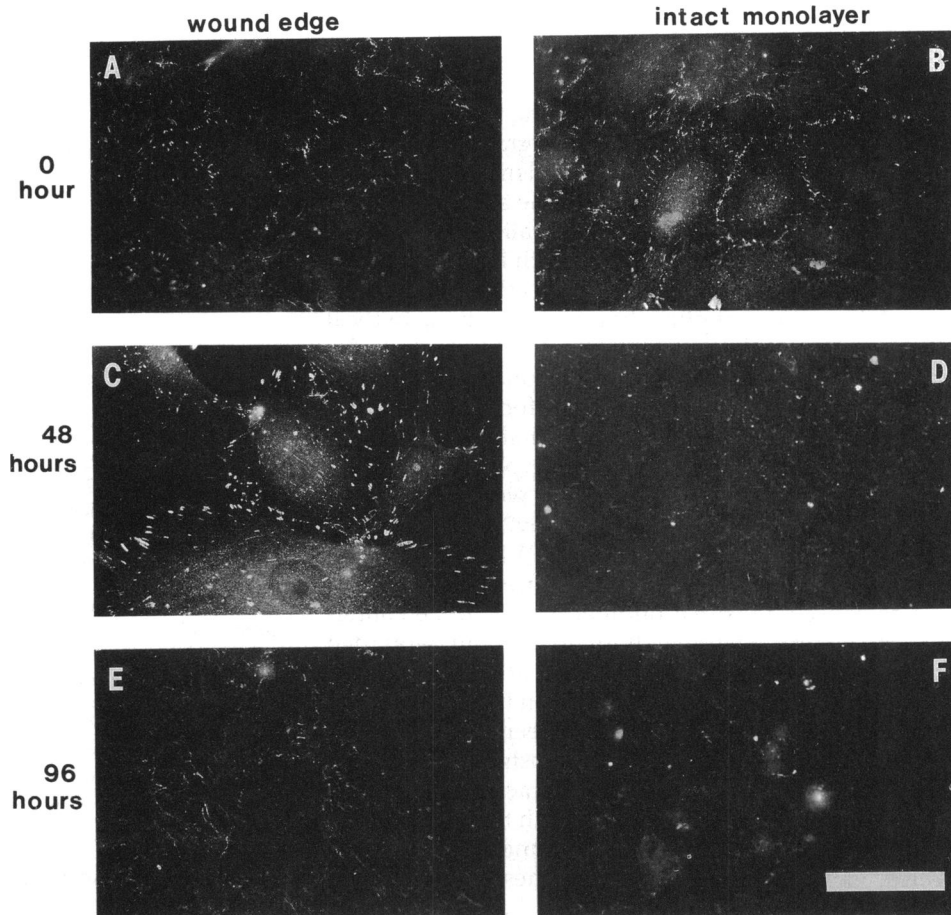
Figure 1. Tyrosine phosphorylation is increased in the focal adhesions of migrating endothelial cells. Confluent monolayers of HUVEC cultured on fibronectin-coated glass coverslips were wounded with a pipette tip and stained for immunofluorescence microscopy with an anti-phosphotyrosine antibody. Coverslips were stained at the time of wounding (0 h, A and B), at 48 h (C and D), and 96 h (E and F) after wounding. A, C, and E depict cells adjacent to the wound, and B, D, and F depict cells distant from the wound breach in the quiescent portion of the monolayer. Phosphotyrosine is prominent in the focal adhesions of the actively migrating cells in C but barely detectable in quiescent cells distant from the wound. The microscopic field is centered over the wounded area in C and E. Bar, 50 μm.

Immunoprecipitation

Cells were lysed by scraping in TBS containing 1 mM Na orthovanadate, 10 μg/ml leupeptin, 0.1% Triton X-100, and 0.1% Na deoxycholate, and the lysates were clarified by centrifugation at 100 000 × g for 30 min. Lysate volumes were normalized for equal protein content using Coomassie blue-staining of sample aliquots subjected to SDS-PAGE. pp125^{FAK} was immunoprecipitated by incubating lysates with antibody 2A7 at 4°C for a minimum of 90 min. Rabbit anti-mouse IgG (Jackson ImmunoResearch) bound to protein A sepharose was then added to these samples and incubated with end over end mixing for a minimum of 90 min at 4°C. Beads were sedimented by brief centrifugation 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.

In Vitro Kinase Activity Assays

pp125^{FAK} that had been immunoprecipitated from cell lysates and bound to protein A sepharose as above was washed with lysis buffer and then with TBS before transfer to 3 mM MnCl$_2$ with 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) pH 7.46. The beads were then incubated with 10 μCi per sample ^{32}P-labelled γATP (New England Nuclear) at room temperature for 20 min to examine pp125^{FAK} autophosphorylation. Samples were then boiled for 3 min with Laemmli sample buffer containing 1 mM sodium orthovanadate and subjected to SDS-PAGE on a 10% polyacrylamide gel with a bisacrylamide concentration of 0.13%. These gels were then stained with Coomassie blue, dried, and exposed to x-ray film and an intensifying

screen at −70°C for autoradiography. Some gels were subjected to alkaline hydrolysis with 1 N KOH at 55°C for 1 h before restaining with Coomassie blue, drying, and autoradiography.

RESULTS

Tyrosine Phosphorylation in Migrating Endothelial Cells

An in vitro model of endothelial cell response to mechanical wounding was used to study the pattern of tyrosine phosphorylation in migrating endothelial cells. Confluent monolayers of HUVEC cultured on fibronectin-coated surfaces were wounded by scraping the cells from the substratum with a pipette tip generating an ~1-mm cell-free path. The distribution of phosphotyrosine in cells migrating into the wound breach was then studied at various time intervals by immunofluorescence microscopy, using the monoclonal antibody py20 (Figure 1). To obtain comparable data from all fields, photographic exposure and development times were identical throughout. Phosphotyrosine was elevated at the edge of the wound in cells that were actively migrating into the cleared area (Figure 1C) (the direction of migration is upward). This observation was noted in

migrating cells as early as 6 h after wounding. The pattern of staining was consistent with elevated tyrosine phosphorylation in focal adhesions at the cell periphery. The elevated phosphotyrosine-staining pattern was not seen beyond three cells from the migrating cell front. The remainder of the monolayer demonstrated low background levels of phosphotyrosine staining even at 48 h after wounding, when the migration process was at its peak. Confluent monolayers, both before and after wounding (Figure 1, B and E, respectively), revealed only a faint pattern of phosphotyrosine staining in focal adhesions. These data are consistent with previous descriptions of increased tyrosine phosphorylation in the newly forming, peripherally located focal adhesions of freshly plated fibroblasts (Burridge *et al.*, 1992b).

The proteins containing increased phosphotyrosine in actively migrating endothelial cells were analyzed by immunoblotting with ^{125}I-labeled py20 (Figure 2A). Multiple wounds were made in HUVEC monolayers with a comb-like device constructed from nylon bristles so as to cause a maximal number of cells to be contiguous to wound margins. Both intact and wounded monolayers were extracted 48–96 h after the wounds were made. The predominant band on these anti-phosphotyrosine autoradiographs was seen at ~120–130 kDa, and an increase in phosphotyrosine was noted in this molecular weight range in the extract of the multiply wounded monolayer as compared with that of the intact control monolayer (Figure 2A). Immunoblot analysis of lysates with enhanced chemiluminescence has not yielded the detection of additional bands. The time course and the ratio of wounded to intact surface area of the monolayer were critical for these experiments, because the increase in tyrosine phosphorylation present in the migrating cell population was diluted by normal phosphotyrosine levels in unaffected cells in large islands of the intact monolayer surface. Western blots of extracts of multiply wounded HUVEC monolayers that had either very few wounds or less time to mobilize cells revealed less phosphotyrosine activity. To achieve a quiescent state, monolayers were harvested 7 d after feeding the cells with fresh serum-containing medium. Intact monolayers that had been more recently fed with fresh serum-containing medium, endothelial cell growth factor, and heparin exhibited higher levels of tyrosine phosphorylation.

We considered the possibility that pp125^{FAK} was one of the proteins with an increase in tyrosine phosphorylation during HUVEC migration. To determine whether a change in pp125^{FAK} expression accompanied the response to wounding, whole cell lysates from intact and wounded monolayers were immunoblotted with anti-pp125^{FAK} antibody. Lysates were normalized for protein concentration before loading on the gel. Equivalent amounts of pp125^{FAK} were found in both intact and wounded monolayers (Figure 2B). pp125^{FAK} was immunoprecipitated from lysates with equal pp125^{FAK}
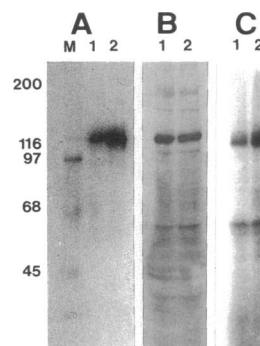
Figure 2. Increased pp125^{FAK} kinase activity is observed in migrating endothelial cells. Cell lysates of intact (lane 1) and multiply wounded (lane 2) HUVEC monolayers cultured on fibronectin were electrophoresed on polyacrylamide gels, transferred to nitrocellulose, and blotted with ^{125}I-py20 (A). Protein loads had been normalized using Coomassie blue-stained gels. Marker proteins (^{125}I-labeled in the left panel) are shown in the lane labeled M. The molecular masses of marker proteins are indicated in kDa to the left of the autoradiograph. Phosphotyrosine is increased in a cluster of dominant bands at 120–130 kDa in the lysate from the wounded monolayer. An anti-pp125^{FAK} immunoblot of whole cell lysates from intact (lane 1) and multiply wounded (lane 2) HUVEC monolayers is shown in B. Equal amounts of pp125^{FAK} were found in the two cell populations. pp125^{FAK} was then immunoprecipitated from parallel lysates and used in in vitro kinase assays with ^{32}P-labeled γATP (C). Autophosphorylation was used as an indicator of kinase activity and was increased in immunoprecipitates from multiply wounded monolayers (C, lane 2) as compared with that of intact controls (C, lane 1).

content, and the immunoprecipitates were incubated with ^{32}P γ-ATP to examine pp125^{FAK} autophosphorylation (Figure 2C). Autophosphorylation of pp125^{FAK} was used as a measure of pp125^{FAK} kinase activity (Lipfert *et al.*, 1992; Schaller *et al.*, 1992). Increased pp125^{FAK} kinase activity was noted in the immunoprecipitates from wounded monolayers as compared to those from intact controls. This increase was quantified using scintillation counting to determine the amount of ^{32}P incorporated into the bands corresponding to pp125^{FAK} that had been excised from the dried gel. A 72% increase in ^{32}P incorporation activity was seen in the band from the multiply wounded monolayer as compared to that from the intact control.

Immunofluorescence microscopy was then used to study the pattern of pp125^{FAK} distribution in migrating and in freshly plated HUVEC (Figure 3, A and B, respectively). To assess the migrating condition, confluent HUVEC monolayers were established on fibronectin-coated coverslips, and migration was induced by removing the cells from one-half of the coverslip with a teflon policeman. Twelve hours after wounding, migrating cells were stained with the monoclonal antibody 2A7 (Figure 3A). Other HUVEC were plated on fibronectin in serum-free conditions for 1 h (Figure 3B). Staining for pp125^{FAK} was localized to a focal adhesion pattern in both migrating and spreading HUVEC. Focal adhesions staining for pp125^{FAK} were seen in both the
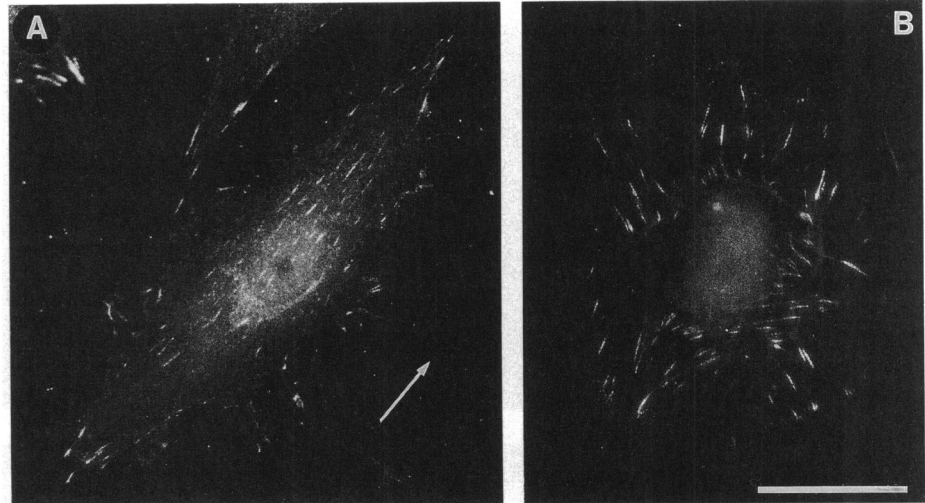
Figure 3. pp125^FAK is localized to focal adhesions in endothelial cells during migration and spreading. HUVEC plated on fibronectin were wounded after growth to confluence (A) or allowed to spread for 1 h in serum-free medium (B). Cells were stained for immunofluorescence with monoclonal anti-pp125^FAK antibody. Staining in a pattern consistent with focal adhesions is seen in both panels. The arrow indicates the direction of cell migration. Bar, 10 μm.

advancing lamellipodia and the trailing tails of migrating cells (Figure 3A). Actively spreading HUVEC exhibited the most prominent pp125^FAK staining in focal adhesions near the cells' periphery (Figure 3B).

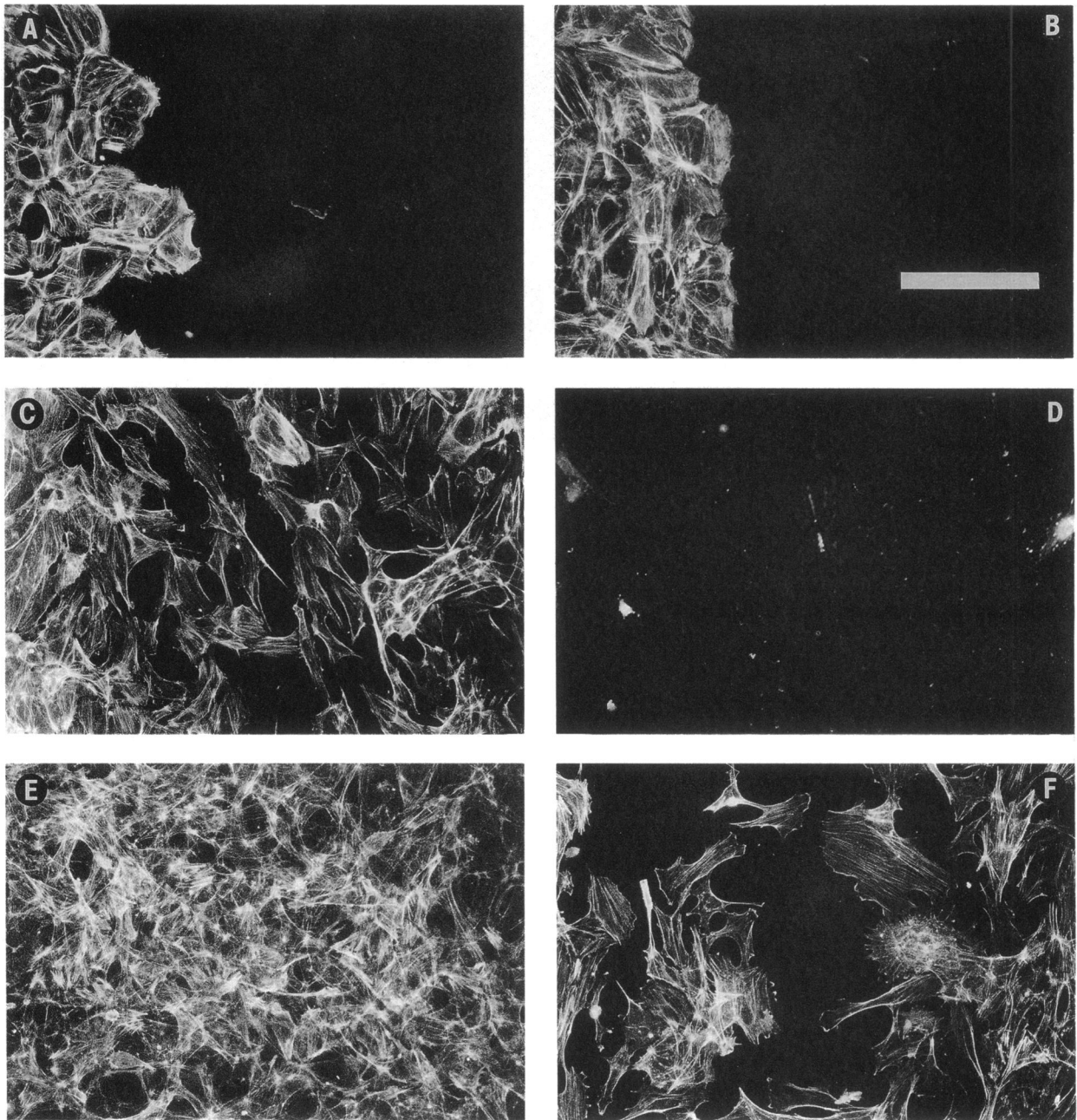### Tyrphostin Inhibition of Endothelial Cell Migration

To study the functional importance of the observed increase in tyrosine phosphorylation in new focal adhesions of actively migrating HUVEC, tyrphostins were assayed for their effect on the rate of wound closure in the wound healing model described above (Figure 4). Tyrphostins were chosen because of their specific inhibition of tyrosine kinases and their low cytotoxicity (Levitski, 1990). Confluent HUVEC monolayers on fibronectin-coated surfaces were wounded with pipette tips. Monolayers were untreated (Figure 4, A, C, and E) or pretreated for 24 h with and maintained in the presence of tyrphostin AG 213 (100 μM, solubilized in DMSO) (Figure 4, B, D, and F) and stained with rhodamine-conjugated phalloidin at various time points after wounding. Tyrphostin was added to treated monolayers on the day of wounding, and this treatment was not augmented on subsequent days. The initial wound breach measured ∼1 mm in width (time 0, Figure 4, A and B). Untreated HUVEC had migrated into the center of the wound breach by 48 h (Figure 4C). During the same time period, tyrphostin-treated cells had only migrated ∼150 μm (Figure 4D). The center of the wound breach in the tyrphostin-treated monolayer is shown in Figure 4D, and a few migrating cells are just seen at the edges. At 96 h, the untreated cells had thoroughly covered all traces of the wound path, whereas the leading cells of the tyrphostin-treated cultures were just entering the central region of the wound breach (Figure 4, E and F, respectively). Pretreatment of monolayers with lower concentrations of AG 213 (25, 50, and 75 μM) produced partial and incremental

inhibition of HUVEC migration. Observations of DMSO-treated HUVEC monolayers did not differ from untreated controls. Herbimycin A (875 nM) and tyrphostin AG 808 (100 μM) also inhibited wound closure. Within 48 h of tyrphostin treatment, a relative paucity of stress fibers and focal adhesions was seen in migrating HUVEC. After 96 h of incubation in the presence of tyrphostin, stress fibers were again seen in migrating cells (Figure 4F). Twenty-four hours of tyrphostin pretreatment had no detectable effect on the stress fibers seen in intact monolayers (Figure 4B).

Tyrphostin Inhibition of Focal Adhesion and Stress Fiber Formation

Tyrphostin effects on focal adhesion formation and stress fiber assembly were then studied in freshly plated HUVEC (Figure 5). To avoid confounding effects of growth factors and extracellular matrix proteins contained in serum, HUVEC were plated on fibronectin for 1 h in medium free of serum (and without heparin and growth factors), in the absence (Figure 5, A, C, and E) or presence (Figure 5, B, D, and F) of tyrphostin AG 213 (100 μm, solubilized in DMSO). HUVEC were pretreated with tyrphostin for some experiments. Cells were stained for immunofluorescence microscopy with antibodies against phosphotyrosine (Figure 5, A and B) and talin (Figure 5, C and D), and with rhodamine-conjugated phalloidin (Figure 5, E and F). Untreated HUVEC revealed a well spread morphology with a well defined focal adhesion pattern when stained with anti-phosphotyrosine antibody. This correlated with the pattern seen for talin (A and C). Many tyrphostin-treated cells, however, were not well spread. Both phosphotyrosine and talin staining were confined to a rim of peripheral brightness, and a focal adhesion pattern was not revealed (B and D). Phalloidin staining demonstrated an array of well developed stress fibers

Figure 4. Tyrphostin AG 213 inhibits endothelial cell wound healing. Untreated HUVEC monolayers (A, C, and E) and monolayers pretreated with and maintained in the presence of 100 μM tyrphostin AG 213 (B, D, and F) were wounded with a pipette tip to generate a 1-mm strip cleared of cells. Coverslips were stained with rhodamine-conjugated phalloidin to visualize cells at the time of wounding (A and B), at 48 h (C and D), and at 96 h (E and F) after wounding. C–F were photographed in the center of the wound breach. Tyrphostin treatment delayed the closure of the wound breach. Bar, 150 μm.

in untreated HUVEC that contrasted sharply with the pattern of reduced stress fibers in the tyrphostin-treated cells (Figure 5, E and F). HUVEC treated with 25 and 50 μM tyrphostin AG 213 demonstrated partial inhibition of stress fiber assembly as compared with cells treated with 100 μM AG 213. DMSO-treated cells re-

vealed staining patterns that were similar to those of the untreated controls.

Inhibition of $pp125^{FAK}$ Kinase Activity

Given the increased kinase activity of $pp125^{FAK}$ in wounded HUVEC monolayers shown in Figure 2, we
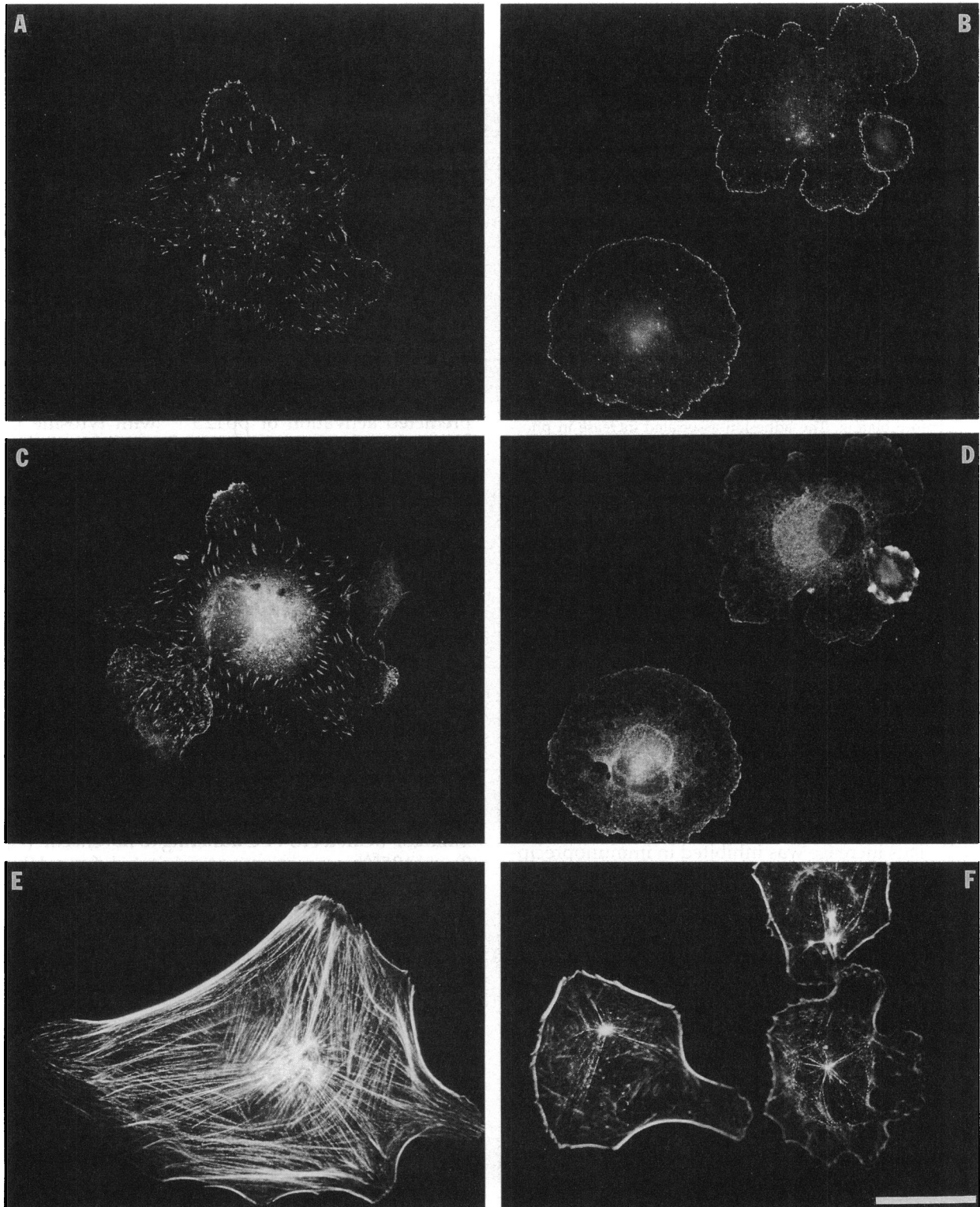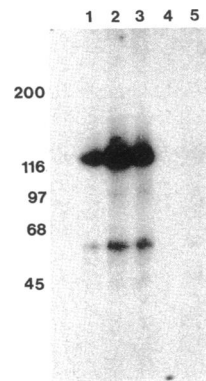
Figure 5. Tyrphostin inhibition of focal adhesion and stress fiber formation in HUVEC. Untreated HUVEC (A, C, and E) or HUVEC plated in the presence of 100 μM tyrphostin AG 213 (B, D, and F) were plated in serum-free medium on fibronectin for 1 h. Cells were stained for immunofluorescence with anti-phosphotyrosine antibodies (A and B), anti-talin antibodies (C and D), or with rhodamine-conjugated phalloidin (E and F). Bar, 10 μm.
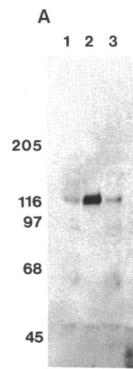
Figure 6. Tyrphostin AG 213 inhibits the adhesion-associated tyrosine phosphorylation of pp125^{FAK}. (A) Anti-phosphotyrosine antibody was used to immunoblot pp125^{FAK} immunoprecipitates from HUVEC kept in suspension for 1 h (lane 1) or plated on fibronectin for 1 h in the absence (lane 2) or presence (lane 3) of tyrphostin AG 213. Tyrphostin-treated HUVEC were preincubated with the inhibitor for 24 h before plating. The adhesion-associated increase in phosphorylation on tyrosine in pp125^{FAK} was inhibited by AG 213. Molecular masses of marker proteins are indicated at the left of the immunoblot. (B) An anti-pp125^{FAK} immunoblot of pp125^{FAK} immunoprecipitates from HUVEC is shown. Cells were plated on fibronectin in the absence (lane 1) or presence (lane 2) of tyrphostin AG 213. Tyrphostin treatment has not altered pp125^{FAK} levels.

Figure 7. Effect of inhibitors on isolated pp125^{FAK} kinase activity. pp125^{FAK} was immunoprecipitated and used in in vitro kinase assays with ^{32}P-labeled γATP. Autophosphorylation was used as an indicator of kinase activity. pp125^{FAK} was immunoprecipitated from HUVEC that were either kept in suspension for 1 h (lane 1) or plated for 1 h on fibronectin in serum-free medium (lanes 2–5). Immunoprecipitates were either untreated (lanes 1 and 2) or treated with herbimycin A (875 nM, lane 3), tyrphostin AG 213 (100 μM, lane 4), or tyrphostin AG 808 (100 μM, lane 5). Molecular masses of marker proteins are indicated at the left of the autoradiograph.

examined the effects of tyrphostin on pp125^{FAK} phosphorylation (Figure 6a). pp125^{FAK} was immunoprecipitated from HUVEC kept in suspension for 1 h (lane 1) or plated on fibronectin for 1 h in the absence (lane 2) or presence (lane 3) of tyrphostin AG 213 (100 μM) and then transferred to nitrocellulose and blotted with HRP-conjugated py20. Increased phosphorylation on tyrosine was noted in the pp125^{FAK} immunoprecipitated from HUVEC that had adhered to fibronectin as compared to that from cells kept in suspension (Figure 6a, lanes 1 and 2). This increase was inhibited in immunoprecipitates from HUVEC that had been plated on fibronectin in the presence of AG 213 (Figure 6a, lane 3). Tyrphostin pretreatment of HUVEC was not, however, associated with alteration of pp125^{FAK} levels (Figure 6b).

We then studied the direct effects of tyrosine kinase inhibitors on isolated pp125^{FAK} kinase activity. For these experiments pp125^{FAK} immunoprecipitates from untreated HUVEC were incubated with ^{32}P-labeled γATP in the presence of tyrosine kinase inhibitors (Figure 7). Untreated HUVEC were kept in suspension (lane 1) or plated on fibronectin for 1 h in serum-free medium (lanes 2–5). Isolated pp125^{FAK} immunoprecipitates were untreated (lanes 1 and 2) or were treated with herbimycin A (875 nM, lane 3), tyrphostin AG 213 (100 μM, lane 4), or tyrphostin AG 808 (100 μM, lane 5). pp125^{FAK} immunoprecipitated from HUVEC kept in suspension showed decreased kinase activity as compared to the pp125^{FAK} from HUVEC that had adhered to fibronectin (lanes 1 and 2). This observation was not altered when the gel was incubated with 1 N KOH for 1 h at 55°C,

indicating that the increased signal represented phosphorylation on tyrosine. This finding correlates with the predicted activation of pp125^{FAK} with tyrosine phosphorylation (Schaller *et al.*, 1992) and with the observed increase in phosphotyrosine on pp125^{FAK} associated with integrin-mediated cell adhesion (Burridge *et al.*, 1992b; Guan and Shalloway, 1992; Hanks, *et al.*, 1992; Kornberg *et al.*, 1992; Lipfert *et al.*, 1992; Romer *et al.*, 1992) (Figure 6, above). Both tyrphostins AG 213 and 808 completely abrogated kinase activity (Figure 7, lanes 4 and 5), whereas herbimycin A only slightly inhibited pp125^{FAK} kinase activity (Figure 7, lane 3). A dose-dependent inhibition of pp125^{FAK} kinase activity by tyrphostin AG 213 was noted (Figure 8). This correlates with our observations of dose-dependent inhibition of HUVEC migration and cytoskeletal organization by tyrphostin AG 213.

To assess the effects of tyrosine kinase inhibitors on pp125^{FAK} activity in intact cells, we then investigated the activity of pp125^{FAK} isolated from tyrosine kinase inhibitor-treated HUVEC adhering to fibronectin (Figure 9). pp125^{FAK} was immunoprecipitated from HUVEC monolayers plated on fibronectin in serum-free medium. HUVEC treated with inhibitors were pretreated with the respective agent for 24 h before plating on fibronectin. The cells were untreated (lane 1) or exposed to
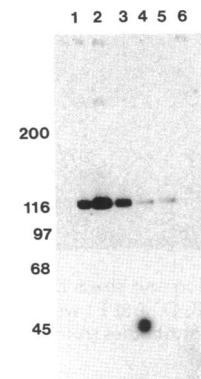
Figure 8. Dose-dependent tyrphostin inhibition of pp125^{FAK} kinase activity in vitro. pp125^{FAK} was immunoprecipitated from HUVEC either kept in suspension for 1 h (lane 1) or plated for 1 h on fibronectin in serum-free medium (lanes 2–6). Immunoprecipitates were untreated (lanes 1 and 2) or treated with increasing concentrations of tyrphostin AG 213 in the presence of ^{32}P-labeled γATP. The tyrphostin concentrations used were 25 μM (lane 3), 50 μM (lane 4), 75 μM (lane 5), and 100 μM (lane 6). Molecular masses of marker proteins are indicated at the left of the autoradiograph.

Figure 9. Inhibition of pp125^FAK kinase activity in HUVEC. Autophosphorylation of pp125^FAK immunoprecipitated from lysates of HUVEC plated in serum-free medium for 1 h on fibronectin was examined using ^{32}P-labeled γATP. This autoradiograph depicts the activity of pp125^FAK immunoprecipitated from untreated HUVEC (lane 1), and HUVEC treated with herbimycin A (875 nM, lane 2), tyrphostin AG 213 (100 μM, lane 3), or tyrphostin AG 808 (100 μM, lane 4). All of the treated groups were pretreated with the respective inhibitor for 24 h and then plated in its presence. Molecular masses of marker proteins are indicated at the left of the autoradiograph.
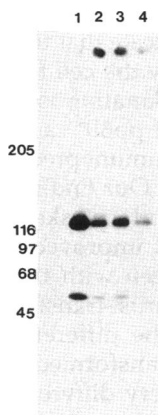
herbimycin A (875 nM, lane 2), tyrphostin AG 213 (100 μM, lane 3), or tyrphostin AG 808 (100 μM, lane 4). Treatment with each of these tyrosine kinase inhibitors resulted in diminished autophosphorylation of pp125^FAK as compared to that of pp125^FAK from untreated HUVEC controls.

The reversibility of the tyrphostin inhibition of pp125^FAK kinase activity in HUVEC was investigated (Figure 10). HUVEC were incubated with AG 213 (100 μM) for 24 h, and the cells were then incubated in the absence of the inhibitor for various time intervals before replating on fibronectin in inhibitor-free conditions. Kinase activity was assayed in pp125^FAK immunoprecipitates from cells incubated for 12- and 24-h tyrphostin-free intervals after treatment (Figure 10, lanes 3 and 4), as well as from untreated HUVEC (Figure 10, lane 1), and from HUVEC that were preincubated with and plated in the presence of AG 213 without a tyrphostin-free interval (Figure 10, lane 2). The diminished pp125^FAK kinase activity associated with tyrphostin AG 213 treatment was noted to return to approximately baseline levels after a 12-h tyrphostin-free incubation (Figure 10, lane 3). Shorter inhibitor-free incubations were associated with incomplete recovery of pp125^FAK kinase activity possibly indicating a prolonged time for the inhibitor to exit the cells. Recovery of cell migration and stress fiber assembly were also studied in tyrphostin-treated HUVEC that were incubated in tyrphostin-free conditions for 18 h. Migration was normal in HUVEC monolayers wounded after this tyrphostin-free interval. Similarly, cytoskeletal assembly was normal in HUVEC plated on fibronectin after the same time interval without tyrphostin.

DISCUSSION

We have used two different model systems to study endothelial cell interactions with extracellular matrix. Both in cells migrating into wounds and in cells freshly plated on fibronectin, we observed increased tyrosine phosphorylation in focal adhesions. Immunoblots of

HUVEC lysates revealed a prominent phosphotyrosine-containing band at 120–130 kDa that was increased in migrating cells. One of the proteins in this cluster is pp125^FAK, which exhibits increased kinase activity in HUVEC migrating into wounds. This finding is consistent with other published observations, including the description of pp125^FAK as a focal adhesion constituent in fibroblasts (Hanks et al., 1992; Schaller et al., 1992), and the activation of pp125^FAK kinase activity during integrin-mediated cell adhesion (Guan and Shalloway, 1992). Our finding that HUVEC required a 7-d incubation without exposure to fresh serum and growth factors to achieve a quiescent state is consistent with others' experience with the induction of quiescence in Swiss 3T3 cells (Rozengurt and Sinnett-Smith, 1983) and with the level of pp125^FAK tyrosine phosphorylation in those cells (Zachary et al., 1992). Recent insights from digitized real time imaging of focal adhesions in confluent endothelial cell monolayers indicate that remodeling of these structures may occur in quiescent cells (Davies et al., 1993). This dynamic state of focal adhesions could account for the low level of pp125^FAK kinase activity that persists in intact HUVEC monolayers, as seen in Figure 2C, lane 1. Additional focal adhesion proteins become tyrosine phosphorylated during endothelial cell adhesion to extracellular matrix. For example, we have also detected elevated phosphotyrosine in paxillin immunoprecipitated from HUVEC that were freshly plated and forming new focal adhesions on fibronectin (Romer, unpublished data). The fact that a band corresponding to paxillin is not seen in Figure 2 correlates with relatively low levels of paxillin in HUVEC.
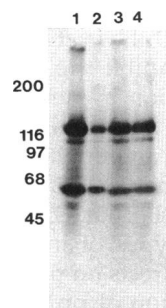
Figure 10. Recovery of pp125^FAK kinase activity after tyrphostin treatment. HUVEC were plated on fibronectin for 1 h in serum-free medium in the absence (lanes 1, 3, and 4) or presence (lane 2) of tyrphostin AG 213 (100 μM). HUVEC for the samples in lane 2 were preincubated with tyrphostin AG 213 for 24 h before plating. Twelve- and 24-h inhibitor-free recovery periods (after AG 213 pretreatment) were used for HUVEC in lanes 3 and 4 respectively. pp125^FAK was then immunoprecipitated from lysates of these cells and assayed for kinase activity by autophosphorylation with ^{32}P-labeled γATP. Recovery of activity is seen in the pp125^FAK from HUVEC plated after a tyrphostin-free interval of 12 or more hours. Molecular masses of marker proteins are indicated at the left of the autoradiograph.

We have also demonstrated that certain tyrosine kinase inhibitors prevented the tyrosine phosphorylation of pp125^{FAK} and interfered with its kinase activity at concentrations that inhibited the cytoskeletal organization and motility of HUVEC. These observations suggest that the activation of this focal adhesion kinase may be necessary for the cytoskeletal organization accompanying HUVEC spreading and migration. Thus, in the absence of elevated levels of pp125^{FAK} activity, there would be insufficient formation of focal adhesions to support cell migration. This scenario is consonant with the role of pp125^{FAK} that is being defined in the work of other investigators. Thus, the tyrosine phosphorylation of pp125^{FAK} has been implicated as an important event in cell spreading (Pelletier *et al.*, 1992), in cytoskeletal organization during cell adhesion (Burridge *et al.*, 1992b; Romer *et al.*, 1992), and in the cytoskeletal organization that accompanies cytokine-stimulated neutrophil adhesion to fibrinogen (Fuortes *et al.*, 1993). Recent studies have also correlated increased levels of pp125^{FAK} expression with neoplasia and metastasis in human colon and breast epithelial cancers in vivo (Weiner *et al.*, 1993). These data suggest that pp125^{FAK} may have a role in the behavior and invasiveness of tumor cells.

Our data from the use of specific tyrosine kinase inhibitors with two different mechanisms of action suggest that it is the inhibition of tyrosine kinase activity, rather than any other effect of these agents, that is responsible for the reported observations. In addition, the use of these two different classes of inhibitors may assist in probing the series of events leading to pp125^{FAK} activation. Tyrphostins are competitive inhibitors of tyrosine kinases that have been engineered to mimic the structure of a tyrosine residue in a peptide chain (Levitski, 1990). They do not affect the activity of protein kinase A, protein kinase C, serine kinases, or threonine kinases (Levitski, 1990; Dvir *et al.*, 1991). Tyrphostins have been noted to inhibit the induction of pp125^{FAK} tyrosine phosphorylation by neuropeptides (Seckl and Rozengurt, 1993). The benzoquinonoid ansamycin herbimycin A, however, is an inhibitor of Src-family kinases with a complex mechanism of action, involving the transfer of a sulfhydryl group (Uehara *et al.*, 1989). We note that these two types of inhibitors also have demonstrably different effects on pp125^{FAK} kinase activity. The complete abrogation of pp125^{FAK} autophosphorylation by tyrphostin AG 213 or AG 808 treatment of isolated pp125^{FAK} immunoprecipitates contrasts sharply with the slight inhibition associated with herbimycin A treatment (Figure 7). Tyrphostin and herbimycin A treatment have much more comparable effects on pp125^{FAK} kinase activity when the inhibitors are used on whole cells (Figure 9). This may indicate that herbimycin A inhibits another kinase that phosphorylates and activates pp125^{FAK} after integrin occupancy in the intact cell. Alternatively, the incomplete action of her-

bimycin A in isolated enzyme systems in vitro may indicate that the inhibitor must undergo some metabolism by the cell to become completely active. This latter explanation is consistent with the incomplete inhibition of pp60^{src} activity by herbimycin A that is observed in immunoprecipitation kinase assays (Uehara *et al.*, 1986).

Our findings that tyrosine kinase inhibitors interfere with cytoskeletal organization contrast with the findings of improved cell spreading and stress fiber formation seen with the use of similar agents on Rous sarcoma virus–transformed rat kidney cells (Uehara *et al.*, 1985). The different effects of tyrosine kinase inhibitors in transformed and normal cells may be explained by the very different levels of tyrosine kinase activity in the two populations. The abnormally high and unregulated expression of pp60^{v-src} in these transformed cells may interfere with orderly protein interactions and specific sequences of tyrosine phosphorylation events that are essential for normal cytoskeletal assembly. Recent work on modulation of tyrosine phosphatase activity in v-src–transfected NIH 3T3 cells supports this view (Woodford-Thomas *et al.*, 1992). Increased phosphatase expression and decreased pp60^{v-src} kinase activity in this system was associated with more normal cytoskeletal organization and cell morphology. In the nontransformed human vascular endothelial cells studied here, treatment with tyrosine kinase inhibitors was likely sufficient to abolish the essential levels of kinase activity needed for normal cytoskeletal function.

Our observation that migrating endothelial cells exhibit well formed focal adhesions differs from descriptions of focal adhesions as typical features of a stationary, well spread phenotype (Couchman and Rees, 1979; Kolega *et al.*, 1982). The contrast in these results may reflect differences in cell type and extracellular matrix environment, but we suspect that this difference is mostly attributable to the techniques used to image focal adhesions. The earlier studies used the definition of a focal adhesion as a structure appearing black or dark gray by interference reflection microscopy, indicating a separation of 10–15 nm between the cell surface and the substratum. Focal adhesions are more frequently defined by immunofluorescence, using antibodies against constituent proteins that label focal adhesion components (Burridge *et al.*, 1988). These two techniques usually coincide in the identification of focal adhesions. We have noted, however, that in cells freshly plated on extracellular matrix proteins focal adhesions are sometimes detected by antibody staining but not by interference reflection microscopy, indicating that the separation between the cell surface and substratum at these sites is greater than ~15 nm (Burridge, unpublished data). Similarly, fibroblasts migrating on laminin revealed focal adhesions upon examination with immunofluorescence but did not exhibit the characteristic black plaques by interference reflection microscopy (Regen and Horwitz, 1992). These investigators also

demonstrated the dynamic turnover of focal adhesion constituents in migrating fibroblasts (Regen and Horwitz, 1992). It is noteworthy that immobilization of the focal adhesion protein talin in fibroblasts by microinjection of specific antibodies resulted in the inhibition of both focal adhesion formation and cell migration (Nuckolls et al., 1992). A similar result was found in this study, in which tyrosine kinase inhibitors blocked both focal adhesion formation and HUVEC migration.

Many processes affect or participate in endothelial cell migration, including growth factors, proteolytic enzymes, chemotactic peptides, and inflammatory cytokines. It will be important to determine the relationship of pp125^{FAK} to these agents. Insulin-like growth factor 1, platelet-derived growth factor (via β receptors), basic fibroblast growth factor, and scatter factor (hepatocyte growth factor) all stimulate endothelial cell migratory responses (Bussolino et al., 1992; Eriksson et al., 1992; Nakao-Hayashi et al., 1992; Pepper et al., 1992). Many of the cellular effects of these growth factors are mediated by receptor-type tyrosine kinases. Interactions of the tyrosine kinase inhibitors used in this current work with receptor kinases will need to be investigated. Inflammatory cytokines, including interleukins 1, 6, and 8, tumor necrosis factor-α, and transforming growth factor-β, have been demonstrated to increase endothelial cell motility (Pepper et al., 1990; Yang and Moses, 1990; Rosen et al., 1991; Basson et al., 1992; Koch et al., 1992). Some of these proteins also alter the repertoire of HUVEC integrin expression (Defillipi et al., 1992) and rearrange HUVEC cytoskeletal structures (Molony and Armstrong, 1991). Cytokines that impact upon endothelial cell motility through the alteration of integrin-mediated adhesiveness may also affect pp125^{FAK} activity.

The mechanism by which integrin receptor occupancy is coupled to the adhesion-associated tyrosine phosphorylation of pp125^{FAK} remains unknown. A 20-min interval of intracellular calcium oscillations has been noted to precede cell spreading and tyrosine phosphorylation during adhesion to fibrinogen in human 293 cells transfected with cDNA for the integrin $\alpha_{IIb}\beta_3$ (Pelletier et al., 1992). Inhibition of these oscillations was found to prevent tyrosine phosphorylation of a 125-kDa protein (presumably pp125^{FAK}), suggesting that pp125^{FAK} phosphorylation may follow the ionic events (Pelletier et al., 1992). Recent HUVEC data indicate that spreading on fibronectin is also associated with an increase in intracellular calcium (Schwartz, 1993). It is possible that integrin occupancy may activate other tyrosine kinases that are upstream of pp125^{FAK} in this signaling pathway. The coprecipitation of an ~58 kDa phosphoprotein with pp125^{FAK} in our Figures 7, 9, and 10 invites speculation that a tyrosine kinase of the Src family may be associated with pp125^{FAK} and may participate in the sequence of tyrosine phosphorylation events during cell adhesion. This speculation is sup-

ported by recent studies that demonstrate stable complexes of pp125^{FAK} with pp60$^{v\text{-}src}$ and pp59^{fyn} (Xing et al., 1993; Cobb et al., 1994). Our current data support but do not prove the hypothesis that pp125^{FAK} has a role in cytoskeletal organization and migration in the endothelium. Other tyrosine kinases may also be involved and are under investigation. The development of more specific tyrosine kinase inhibitors may help elucidate the complex series of tyrosine phosphorylation events that accompanies cell adhesion to extracellular matrix proteins.

ACKNOWLEDGMENTS

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