Integrin \( \alpha_v \beta_5 \)-dependent Serine Phosphorylation of Paxillin in Cultured Human Macrophages Adherent to Vitronectin*

Mark O. De Nichilo and Kenneth M. Yamada

From the Laboratory of Developmental Biology, NIDR, National Institutes of Health, Bethesda, Maryland 20892

The macrophage colony-stimulating factor (M-CSF) is able to induce the expression of the \( \alpha_v \beta_5 \) integrin receptor on the surface of cultured human macrophages (De Nichilo, M. O., and Burns, G. F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2517-2521). In the present study we establish that the adhesion of M-CSF-treated macrophages to vitronectin is mediated by the integrin \( \alpha_v \beta_5 \) and show by indirect immunofluorescence analysis that \( \alpha_v \beta_5 \) and the cytoskeletal protein paxillin localize to focal contacts upon adhesion to vitronectin. Immuno-precipitation and Western blot analysis revealed that M-CSF-treated macrophages do not express focal adhesion kinase (FAK), thereby providing direct evidence for integrin-dependent localization of paxillin to focal contacts in the absence of FAK expression. Investigation of paxillin phosphorylation by two-dimensional phosphoamino acid analysis indicates that paxillin is 99% phosphorylated on serine residue(s) in response to vitronectin adhesion, and only 1% phosphorylated on tyrosine. Stimulation of protein kinase C (PKC) activity with the phorbol ester phorbol 12-myristate 13-acetate enhances paxillin phosphorylation, while two selective inhibitors of PKC, GF109203X and chelerythrine chloride, effectively block the phosphorylation of paxillin induced in response to vitronectin adhesion. Taken together, these data demonstrate that in M-CSF-treated macrophages adherent to vitronectin, paxillin localizes to focal contacts in the absence of FAK expression and is predominantly phosphorylated on serine residue(s) in a PKC-dependent manner.

Interactions with the extracellular matrix (ECM) are known to have profound influence on cell growth and differentiation, as well as migration (1). The receptors that have received most study in this regard belong to a large family of \( \alpha \beta \) heterodimers termed integrins (2). Integrins function as cell surface receptors for a variety of ECM proteins (including vitronectin, fibronectin, laminin, and the collagens) and are thought to transmit signals to the cell upon ligand occupancy, and thereby regulate cell behavior (3-5). The integrin family is now known to consist of over 20 distinct receptors, with the combination of a particular \( \alpha \) and \( \beta \) subunit determining ligand specificity (2). In general, upon binding their specific ligands, the integrins associate with the actin cytoskeleton and organize into structures known as focal contacts (focal adhesions or adhesion plaques) (6). Focal contacts are thought to act not only as structural links between the ECM and the cytoskeleton, but also as sites of signal transduction from the ECM (6). Despite their importance, the underlying molecular mechanisms orchestrating the formation of focal contacts upon integrin ligand remain poorly defined.

FAK (focal adhesion kinase) is a 125-kDa cytoplasmic protein tyrosine kinase that was named for its ability to localize to focal contacts (7, 8). Published data obtained primarily using cultured fibroblasts have shown that engagement of integrins with ECM ligands or cross-linking of cell surface integrins with antibodies leads to a pronounced increase in the tyrosine phosphorylation of FAK (9-12) and a concomitant increase in its intrinsic tyrosine kinase activity in vitro (13, 14). The presence of tyrosine-phosphorylated proteins in focal contacts and the regulation of their tyrosine phosphorylation in response to integrin-mediated cell adhesion has led to the suggestion that FAK plays a central role in regulating focal contact assembly (10).

Paxillin is a 68-kDa vinculin-binding protein that also colocalizes with FAK and integrins to focal contacts (15, 16) and is phosphorylated on tyrosine residues during integrin-mediated adhesion of fibroblasts to ECM substrates (10). Paxillin has been identified as a target substrate for FAK phosphorylation in vitro (17-19). On the basis of their colocalization to focal contacts and coordinate phosphorylation, it has been hypothesized that paxillin phosphorylation is closely coupled to FAK activation (10, 16, 19). Recent evidence, however, suggests that the tyrosine phosphorylation of paxillin mediated by FAK may not be a critical determinant in paxillin localization to focal contacts (20), suggesting that FAK phosphorylation of paxillin may serve an additional function possibly related to cell signaling (19, 20).

The serine/threonine kinase, protein kinase C (PKC) is another regulatory enzyme that has been localized to focal contacts (21, 22). Woods and Couchman (23) previously provided evidence in support of PKC involvement in the regulation of focal contact formation in fibroblasts. While it is well established that compounds that activate PKC such as phorbol 12-myristate 13-acetate (PMA) enhance cell adhesion and spreading on ECM substrates (24-26), the identity of cytoskeletal proteins within focal contacts that serve as targets for PKC-mediated phosphorylation in response to integrin-dependent cell adhesion remain poorly defined. In the present study, we show that in M-CSF-treated macrophages, paxillin localizes to focal contacts in the absence of FAK expression and is predominantly serine-phosphorylated in response to integrin-activated adhesion to vitronectin. PMA stimulation of PKC activity was found to enhance paxillin phosphorylation,
whereas selective inhibitors of PKC activity effectively block the phosphorylation of paxillin induced in response to vitronectin adhesion. These latter results indicate that serine phosphorylation of paxillin observed in response to vitronectin adhesion is thus PKC-dependent.

**EXPERIMENTAL PROCEDURES**

Monocyte Purification and Culture—Human peripheral blood cells were obtained by leukapheresis of normal volunteers at the Department of Transfusion Medicine at the National Institutes of Health. These cells were washed four times with 250 ml of Ca²⁺/Mg²⁺-free Hank’s balanced salt solution containing 2 mM EDTA to reduce platelet contamination, and then layered over a 20-mL lymphoprep cushion (Nyegaard, Oslo) in 50-ml tubes (Falcon, Division of Becton Dickinson, Oxnard, CA). After density sedimentation at 400 × g for 20 min, the monocytes in the mononuclear cell layer were purified by counterflow centrifugal elutriation in a Beckman elutriation system as described previously (27), except that phosphate-buffered saline (PBS) was used in the elutriation procedure. The method outlined above consistently yielded >94% pure monocytes as determined by flow cytometric, morphological, and cytochemical criteria as described (28). Purified monocytes were resuspended in RPMI 1640 supplemented with 2 mM glutamine, 10 mM gentamycin sulfate, and 10% (v/v) fetal bovine serum (heat inactivated). Cells were maintained in a nonadherent state in polystyrene plates (Nunc, Denmark) with several changes of medium containing human recombinant M-CSF (Genzyme, Cambridge MA) at 50 units/ml for 48 h. After 48 h in culture, these cells are referred to as M-CSF-treated macrophages.

**Endotoxin Levels**—Bacterial endotoxin is a potent regulator of leukocyte function (29). To minimize its contamination, all monocyte purification and culturing was performed using sterile disposable plasticware, and only reagents of the highest quality were used. Fetal bovine serum (BioWhittaker, Walkersville, MD) was screened using the Limulus amoeboocyte lysate assay and selected for low endotoxin content (0.5 endotoxin units/ml) and the inability to support monocyte survival in the absence of exogenous stimulus; the M-CSF (purified recombinant cDNA-expressed in yeast) was also shown to contain <0.005 endotoxin unit/ml as assayed by the manufacturer. Phosphate-free RPMI 1640 (Life Technologies, Inc./BRL, Gaithersburg, MD) contained <0.03 endotoxin unit/ml.

**Fibroblast Cell Culture**—Human foreskin fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc./BRL) containing 2 mM glutamine, 50 mM streptomycin, 50 units/ml penicillin, and 10% fetal bovine serum (HyClone, Logan, UT). The cells were isolated and kindly provided by Susan Yamada and were used at cell passages 4–8.

**Attachment**—The monoclonal antibodies (mAbs) used were: LM142 (Chenbron, Tenerua, CA) directed against the α₅ subunit; SZ-21 (Immunotech, Westbrook, ME) which has specificity for the β₁ subunit; B5-VF2 (UBI, Lake Placid, NY) and P1F6 (Life Technologies, Inc./BRL) which recognize the β₃ subunit and α₅β₃ complex, respectively. Monoclonal antibodies against paxillin and FAK were obtained from Transduction Laboratories (Lexington, KY), and antibody VII-F8311 against human vinculin was a gift from Dr. Victor Kotliarenky (CNRS-Institut Curie, Paris). Antibody 3G10 (Boehringer Mannheim) directed against the α-chain of the interleukin-2 receptor was also obtained. Fibroblasts were seeded on 96-well cluster plates (Costar, Cambridge, MA) coated with 100 μg/ml of vitronectin (0.3–100 μg/ml in PBS) at 4°C overnight, and nonadherent cells were removed by gentle washing. Macrophages were harvested, washed twice with RPMI 1640, and resuspended in RPMI 1640 containing 1 μM CaCl₂ and BSA at 2.5 × 10⁶ cells/ml (10% per well) were allowed to adhere for 60 min at 37°C, and nonadherent cells were removed by gentle washing. For phosphorylation studies, poly-L-lysine (Sigma-Aldrich, St. Louis, MO) was coated overnight at 10 μg/ml in PBS. Macrophages were allowed to attach and then washed twice with cold PBS, and solubilized in RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 2 mM EDTA) containing a variety of protease and phosphatase inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM benzamidine trypsin inhibitor, 1 mM sodium fluoride, and 1 mM sodium orthovanadate) for 20 min on ice. The lysates were clarified by centrifugation at 20,000 × g for 15 min at 4°C, and protein concentrations were determined using the biocinchonic acid protein assay kit (Pierce). The lysates were adjusted to equal protein concentrations and volume and precluded overnight at 4°C by incubating end-over-end with protein G-Sepharose beads (Pharmacia, Piscataway, NJ) and 30 mM mouse IgG (Rockland, Gilbertsville, PA). The beads were sedimented by brief centrifugation, and immunoprecipitations were performed by incubating the preclad lysates with end-over-end mixing with the designated antibodies (4 μg/ml) together with protein G-Sepharose for 4 h at 4°C. The beads were washed twice with ice-cold RIPA lysis buffer, then twice with the same buffer now containing 500 mM NaCl. The immunoprecipitated proteins were eluted by boiling in reducing SDS sample buffer (62 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, and bromphenol blue) for 5 min.

**Western Immunoblotting**—Immunoprecipitates were resolved in 7.5% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell) for 2 h at 250 mA. The filters were blocked with blocking buffer (5% BSA in TTBS; 150 mM NaCl, 50 mM Tris, 0.2% Tween 20, pH 7.4) for 3 h at room temperature. Blots were probed with either the aforementioned α-chain antibody or AK-1 mAbs at 1 μg/ml in blocking buffer. After extensive washing at 37°C with several changes of TTBS, the filters were incubated for 45 min with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham) at a 1:3000 dilution in blocking buffer. After washing, immunoreactivity was detected by using the enhanced chemiluminescence (ECL) reaction (Amersham). To assess the tyrosine phosphorylation status of paxillin in response to vitronectin adhesion, blots were probed with horseradish peroxidase-conjugated PY-20 mAb (ICN) at a 1:7000 dilution. Filters were then stripped with 2% SDS, 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8, for 30 min at 70°C, and reprobed for paxillin as described above.

**In Vivo Phosphorylation of Paxillin**—In vivo phosphorylation studies were conducted as described (30), with minor modifications. Nonadherent M-CSF-treated macrophages were harvested and endogenous phosphate levels depleted by washing twice with phosphate-free RPMI 1640, then resuspending the cells in phosphate-free RPMI 1640 containing 1 μg/ml bovine serum albumin (BSA) and incubating for 30 min at 37°C. After phosphate starvation, the cells were biosynthetically labeled by incubating in phosphate-free RPMI 1640 containing 1 μg/ml BSA and 150 μCi/ml [³²P]orthophosphate (9000 Ci/mmol; DuPont NEN) for 90 min at 37°C. Macrophages were then stimulated under the various conditions as described while in the continuous presence of [³²P]orthophosphate. Paxillin phosphorylation was assayed by immunoprecipitation followed by SDS-PAGE and autoradiography. The protein kinase C inhibitors GF109203X (Boehringer Mannheim) and chelerythrine chloride (LC Laboratories, Woburn, MA), when used, were added to the cells during the last 60 min incubation with [³²P]orthophosphate prior to stimulation, and were maintained in the culture medium throughout. Stock solutions of GF109203X (5 μM), chelerythrine chloride (10 μM), and the protein kinase C activator PMA (5 mg/ml) were dissolved in dimethyl sulfoxide. PMA was obtained from Sigma. Protein phosphatase analysis was performed essentially as described by Siegel (30). Autoradiograms were scanned using a laser densitometer (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA) and the results expressed as arbitrary units normalized against background.

**Attachment to Vitronectin**—Vitronectin was purified from human serum according to the method of Yatohgo et al. (31), and its purity was determined by SDS-PAGE and Coomassie Blue staining. For immunofluorescence localization studies, sterile glass coverslips (18 mm) were placed into 12-well plates (Nunc) and coated at 4°C overnight with human vitronectin (50 μg/ml in 0.5 ml of PBS). Cultured macrophages were harvested, washed twice with RPMI 1640, and resuspended in RPMI 1640 containing 1 mM CaCl₂ and BSA at 1.5 × 10⁶ cells/ml (10% per well) were allowed to adhere for 60 min at 37°C, and nonadherent cells were removed by gentle washing. For phosphorylation studies, poly-L-lysine (Sigma-Aldrich, St. Louis, MO) was coated overnight at 10 μg/ml in 4% PBS.

**Indirect Immunofluorescence**—Macrophages were allowed to attach and then washed twice with cold PBS, and then incubated on coated coverslips and stained by indirect immunofluorescence as described (28).

**Adhesion Assays**—Cell adhesion assays were performed as described previously with modifications (32). In brief, sterile tissue culture grade 96-well cluster plates (Costar, Cambridge, MA) were coated with 100 μl/well of vitronectin (0.3–100 μg/ml in PBS) at 4°C overnight, and then washed 3 times with PBS. Macrophages were added to the wells immediately before use. Macrophages were harvested, washed twice with RPMI 1640, and resuspended to 2.5 × 10⁶ cells/ml in RPMI 1640 containing 1 mM CaCl₂ and 1 mg/ml BSA. A 100-μl volume of the cell suspension was added to each well and the plates were incubated for 30 min at 37°C. After the attachment period, nonadherent cells were poured off and residual cells stained with 0.25% (w/v) Rose Bengal dye (Sigma) in PBS for 10 min at room temperature. The wells were then washed twice with PBS and the remaining dye was released by incubating with 100 μl/well 50% ethanol in PBS. Released dye was quanti-
Integrin-dependent Serine Phosphorylation of Paxillin

We reported previously that the colony-stimulating factor M-CSF was able to induce the expression of the \( \alpha_v \) subunit (\( \alpha_v \)) in M-CSF-treated macrophages (28). Taken together, these data indicate that M-CSF-treated macrophages express \( \alpha_v \) only in association with the \( \beta_5 \) subunit.

Integrin \( \alpha_v \beta_5 \) Mediates the Adhesion of M-CSF-treated Macrophages to Vitronectin—Fig. 2A shows that M-CSF-treated macrophages adhere to vitronectin in a dose-dependent manner, with half-maximal adhesion achieved at a coating concentration of 1 \( \mu \)g/ml and reaching a plateau at approximately 10 \( \mu \)g/ml. To investigate whether \( \alpha_v \beta_5 \) was the integrin responsible for mediating the adhesion of M-CSF-treated macrophages to vitronectin, cell adhesion assays were performed in the presence of specific anti-integrin monoclonal antibodies. As shown in Fig. 2B, mAb P1F6 directed against a functional epitope on integrin \( \alpha_v \beta_5 \) substantially inhibited the adhesion of M-CSF-treated macrophages to vitronectin in a dose-dependent manner. In contrast, the non-inhibitory anti-\( \alpha_v \) mAb LM142 which served both as an IgG isotype and ascites control, failed even at the highest concentration to influence the adhesion of M-CSF-treated macrophages to vitronectin. These data demonstrate that the adhesion of M-CSF-treated macrophages to vitronectin is mediated by the integrin \( \alpha_v \beta_5 \).

Integrin \( \alpha_v \beta_5 \) and Paxillin Localize to Focal Contacts on Vitronectin—Evidence for the functional involvement of integrins in cell spreading on a particular substrate can be obtained directly by examining the distribution of the receptors involved. This was accomplished by permitting M-CSF-treated macrophages to adhere to vitronectin for 60 min and examining these cells for the distribution of the \( \beta_5 \) subunit by indirect immunofluorescence analysis. As shown in Fig. 3B, \( \beta_5 \) staining in permeabilized cells was localized to streaks, both peripheral and under the cell body. In these cells, the streaks could be

RESULTS

M-CSF-treated Macrophages Express \( \alpha_v \) Only in Association with \( \beta_5 \)—We reported previously that the colony-stimulating factor M-CSF was able to induce the expression of the \( \alpha_v \beta_5 \) integrin receptor on the surface of cultured human macrophages (28). To confirm and extend these observations, flow cytomeric analyses were performed on M-CSF-treated macrophages using mAbs directed against the \( \beta_5 \) subunit and \( \alpha_v \beta_5 \) complex that were not available at the time of our earlier study. Fig. 1 demonstrates that the fluorescence intensity of cell surface staining for both the \( \beta_5 \) subunit and the \( \alpha_v \beta_5 \) complex were equivalent to the levels observed for the \( \alpha_v \) subunit. In contrast, the anti-\( \beta_5 \) mAb exhibited levels of immunostaining no greater to that of control cells where the primary antibody had been omitted. Our previous study indicated that M-CSF-treated macrophages fail to express the integrin \( \alpha_v \beta_1 \) as was shown by preclearing \( \beta_1 \) from a lysate of radiolabeled cells and demonstrating that subsequent clearance there was no residual \( \alpha_v \) (28). Taken together, these data indicate that M-CSF-treated macrophages express \( \alpha_v \) only in association with the \( \beta_5 \) subunit.

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identified as focal contacts by both vinculin (Fig. 3C) and talin staining (data not shown). Staining with anti-paxillin mAb also revealed the localization of paxillin to focal contacts in cells adherent to vitronectin (Fig. 3D).

Localization of Paxillin to Focal Contacts Is Independent of FAK Expression—FAK is a 125-kDa cytoplasmic protein tyrosine kinase thought to play a central role in orchestrating focal contact formation in response to integrin-mediated cell adhesion to the extracellular matrix (8). In particular, it has been suggested that the localization of paxillin to focal contacts and its phosphorylation on tyrosine are closely coupled to FAK activation (10). A recent report found that human monocytes freshly isolated from peripheral blood fail to express FAK (33). Based on these observations, we examined whether M-CSF, in addition to its ability to regulate \( \alpha_v\beta_3 \) expression, could also regulate the expression of FAK in cultured human macrophages, thereby facilitating the localization of paxillin to focal contacts in response to \( \alpha_v\beta_3 \)-mediated adhesion to vitronectin. Immunoprecipitation and Western blot analysis revealed that while M-CSF-treated macrophages express abundant paxillin, these cells do not express FAK (Fig. 4). As a positive control, FAK could be readily detected in whole cell lysates obtained from human foreskin fibroblasts (Fig. 4). Indirect immunofluorescence localization studies using mAbs and a polyclonal antiserum raised against FAK have also confirmed the absence of FAK in M-CSF-treated macrophages adherent to vitronectin (data not shown). We therefore conclude that the localization of paxillin to focal contacts in M-CSF-treated macrophages adherent to vitronectin occurs independent of FAK expression.

Paxillin Is Predominantly Serine-phosphorylated in Response to Vitronecin Adhesion—In light of the observation that M-CSF-treated macrophages fail to express FAK, experiments were undertaken to determine whether or not paxillin could be tyrosine-phosphorylated in response to vitronectin adhesion. Total cell lysates from M-CSF-treated macrophages that were maintained either in a nonadherent state or allowed to attach and spread on uncoated or vitronectin-coated dishes for 60 min, were immunoprecipitated with a monoclonal anti-paxillin antibody and analyzed by Western immunoblotting using an antiphosphotyrosine monoclonal antibody. As shown in Fig. 5D, paxillin was specifically tyrosine-phosphorylated, albeit at very low levels, in response to vitronectin adhesion. In contrast, tyrosine phosphorylation of paxillin was not observed in nonadherent cells or in cells adherent to plastic. Interestingly, reprobing the blots to confirm equal protein loading revealed the presence of a slower migrating form of paxillin immunoprecipitated from macrophages adherent to vitronectin (Fig. 5D). Indirect immunofluorescence analysis reveals a close association between paxillin localization to focal contacts (Fig. 5B) and the tyrosine phosphorylation and mobility shift of paxillin observed in M-CSF-treated macrophages upon adhesion to vitronectin (Fig. 5D). In contrast, paxillin remains diffusely distributed in cells that attach and spread on uncoated coverslips over the same time period (Fig. 5A).

Zachary et al. (34) reported a similar mobility shift of paxillin in Swiss 3T3 fibroblasts stimulated with bombesin. These authors noted that even though paxillin was strongly tyrosine-phosphorylated in response to bombesin stimulation, inhibition of the PKC pathway either by down-regulation of PKC or treatment with the selective PKC inhibitor GF109203X, as shown in Fig. 5D, paxillin was specifically tyrosine-phosphorylated, albeit at very low levels, in response to vitronectin adhesion. In contrast, tyrosine phosphorylation of paxillin was not observed in nonadherent cells or in cells adherent to plastic. Interestingly, reprobing the blots to confirm equal protein loading revealed the presence of a slower migrating form of paxillin immunoprecipitated from macrophages adherent to vitronectin (Fig. 5D). Indirect immunofluorescence analysis reveals a close association between paxillin localization to focal contacts (Fig. 5B) and the tyrosine phosphorylation and mobility shift of paxillin observed in M-CSF-treated macrophages upon adhesion to vitronectin (Fig. 5D). In contrast, paxillin remains diffusely distributed in cells that attach and spread on uncoated coverslips over the same time period (Fig. 5A).

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with orthophosphate, paxillin was heavily phosphorylated very little orthophosphate was incorporated into paxillin appeared to be a specific integrin-mediated response, because residues in response to whether paxillin could be phosphorylated on serine/threonine phosphorylation. Based on these observations, we examined directly influencing the levels of tyrosine phosphorylation, thus sug-
tion mediated by PMA stimulation was also accompanied by a shif in the relative mobility of paxillin (Fig. 7a), representing phosphopeptides generated by incomplete acid hydrolysis.

blocked this characteristic mobility shift of paxillin without influencing the levels of tyrosine phosphorylation, thus sug-
ting the potential involvement of serine/threonine phosphophorylation. Based on these observations, we examined directly whether paxillin could be phosphorylated on serine/threonine residues in response to αβ₃-mediated adhesion to vitronectin. Fig. 6A shows that in M-CSF-treated macrophages labeled with [³²P]orthophosphate, paxillin was heavily phosphorylated in response to vitronectin adhesion. This phosphorylation appeared to be a specific integrin-mediated response, because very little [³²P]orthophosphate was incorporated into paxillin that was immunoprecipitated from cells either maintained in a nonadherent state or allowed to attach and spread on plastic over the same period of time (Fig. 6A). Two-dimensional phosphoamino acid analysis revealed that 99% of the [³²P]orthophosphate incorporated into paxillin upon adhesion to vitronectin was on serine residue(s) (Fig. 6B). In contrast, phosphorylation on tyrosine residue(s) accounted for only 1% of the total paxillin phosphorylation, a figure that is consistent with the low levels of paxillin tyrosine phosphorylation observed by Western immunoblotting (Fig. 5D). Phosphorylation on threonine residue(s) was not detected by phosphoamino acid analysis under the conditions described (Fig. 6B). These data provide the first evidence for serine phosphorylation of paxillin in response to integrin-mediated adhesion to vitronectin.

PMA Activation of PKC Enhances the Phosphorylation of Paxillin—Cloning and sequence analysis has revealed that paxillin contains six potential consensus target phosphorylation sites for PKC (18, 35). To determine whether direct activation of PKC could enhance paxillin phosphorylation, M-CSF-treated macrophages were labeled with [³²P]orthophosphate and allowed to attach and spread on vitronectin-coated dishes for 60 min in the absence or presence of the PKC activator PMA. As shown in Fig. 7A, PMA stimulation of M-CSF-treated macrophages on vitronectin resulted in a 2.5-fold increase in paxillin phosphorylation as compared to the levels observed in the absence of PMA stimulation. This enhanced phosphorylation mediated by PMA stimulation was also accompanied by a shift in the relative mobility of paxillin (Fig. 7A). Turner et al. (36) recently demonstrated an increase in paxillin tyrosine phosphorylation following stimulation of rat aortic smooth muscle cells with PMA. Anti-phosphotyrosine Western blotting of paxillin immunoprecipitates prepared from lysates obtained from macrophages treated under parallel conditions to those described in Fig. 7A revealed no increase in the level of paxillin tyrosine phosphorylation upon PMA treatment (data not shown), suggesting the increase in phosphorylation mediated by PMA was likely to be on serine/threonine residue(s). Next, we examined the effect of PMA stimulation on the kinetics of paxillin phosphorylation in cells on vitronectin. As shown in Fig. 7B, adhesion of M-CSF-treated macrophages to vitronectin in the absence of PMA stimulation resulted in a time-dependent increase in paxillin phosphorylation, that was first evident within 15 min following cell attachment and began to plateau by 60 min. Stimulation with PMA clearly enhanced the rate and magnitude of paxillin phosphorylation over the same time period (Fig. 7B). The levels of paxillin phosphorylation observed in Fig. 7B closely parallel the time course of adhesion and spreading of M-CSF-treated macrophages to vitronectin under the same conditions (data not shown). PMA stimulation was also found to enhance the recruitment of both integrin α₂β₃ and paxillin to focal contacts. The effect of PMA stimulation on paxillin localization to focal contacts is illustrated in Fig. 8. Indirect immunofluorescence analysis indicates that cells spreading on vitronectin over a 60-min period in the presence of PMA have a greater number of focal contacts containing paxillin as compared to cells that attach and spread on vitronectin in the absence of PMA treatment (Fig. 8).

Inhibition of PKC Activity Blocks α₂β₃-Mediated Serine Phosphorylation of Paxillin—To further examine the involvement of PKC in mediating serine phosphorylation of paxillin in response to vitronectin adhesion, we employed two potent inhibitors of PKC activity, the bisindolylmaleimide GF 109203X and chelerythrine chloride. Both compounds have been reported to be highly selective for PKC, with GF 109203X able to compete for ATP binding (37), while chelerythrine chloride inhibits the catalytic domain of PKC (38). Fig. 9 shows that pretreatment of [³²P]orthophosphate labeled M-CSF-treated macrophages with either GF 109203X (10 μM) or chelerythrine chloride (1 μM) effectively inhibited the serine phosphorylation of paxillin in-

**Fig. 6. Analysis of total paxillin phosphorylation.** A, M-CSF-treated macrophages were labeled with [³²P]orthophosphate as described under “Experimental Procedures,” then maintained either in a nonadherent state for 60 min (lane 1), or allowed to attach and spread on uncoated (lane 2) or vitronectin-coated (lane 3) Petri dishes for 60 min. Cells were solubilized in RIPA lysis buffer, and equal concentrations and volumes of cell lysates were immunoprecipitated using an anti-paxillin mAb. The precipitated immunocomplexes were visualized following SDS-PAGE and autoradiography. B, the phosphorylated band corresponding to paxillin was excised and subjected to two-dimensional phosphoamino acid analysis as described under “Experimental Procedures.” PT (phosphothreonine), PY (phosphoysine), and PS (phosphoserine) indicate the relative migration of the phosphoamino acid standards. The smear running below and to the left of the PY area represents phosphopeptides generated by incomplete acid hydrolysis.

**Fig. 7. Effect of PMA on paxillin phosphorylation.** A, M-CSF-treated macrophages were labeled with [³²P]orthophosphate as described under “Experimental Procedures.” Cells were subsequently maintained either in a nonadherent state for 60 min (lane 1), or allowed to attach and spread on vitronectin-coated dishes for 60 min in the absence (lane 2) or presence of 5 ng/ml PMA (lane 3). To assess paxillin phosphorylation, cells were solubilized in RIPA lysis buffer and equal concentrations and volumes of cell lysates were immunoprecipitated using an anti-paxillin mAb. The precipitated immunocomplexes were visualized following SDS-PAGE and autoradiography. B, M-CSF-treated macrophages were labeled with [³²P]orthophosphate as described under “Experimental Procedures.” Cells were then allowed to attach and spread on vitronectin-coated dishes for timepoints up to and including 60 min in the presence or absence of 5 ng/ml PMA. Paxillin phosphorylation was assessed as described above in A. Autoradiographs were scanned by laser densitometry and results expressed as arbitrary units normalized against background.
ever, the identity of the M-CSF is able to induce expression of the
and chelerythrine chloride. These data demonstrate that
stantially inhibit the low levels of paxillin tyrosine phosphoryl-
mediated phosphorylation of paxillin (Fig. 9). It is also of interest to
confirm by using mAbs to the α5β3 subunit and αvβ5 complex that
these cells adhere to vitronectin in an αvβ5-dependent manner.
Indirect immunofluorescence analysis establishes definitively
the localization of α5β3 to focal contacts, suggesting the surface
expression of α5β3 on cultured human macrophages is a deter-
mining factor in their morphology on vitronectin.
We find that like the integrin αvβ3, paxillin, a vinculin-
binding protein (15, 16) also localizes to focal contacts in M-
CSF-treated macrophages adherent to vitronectin. FAK is
thought to play a central role in the organization of the cytoskeleton as cells adhere to the ECM. In particular, it has been
suggested that the localization of paxillin to focal contacts and
its phosphorylation on tyrosine are tightly coupled to FAK
activation (10). We demonstrate here by immunoprecipitation and Western blot analysis, together with indirect immunofluo-
rescence studies, that M-CSF-treated macrophages do not express FAK. These observations are consistent with previous
reports in mouse bone marrow-derived macrophages (39), and
in human peripheral blood monocytes (33) that demonstrate
the absence of FAK expression at both the RNA and protein
levels, respectively. Our findings and particularly those
described by J uliano’s group (33) are somewhat at variance with
the observations recently reported by Kharbada et al. (40).
These authors reported the presence of FAK protein in human
monocytes freshly isolated from peripheral blood. The reason
for this discrepancy is not known; one distinct possibility, how-
ever, is that FAK observed in these cells is not of monocyte
origin, but is rather derived from contaminating platelet mi-
croparticles that are rich in FAK and are known to bind to the
surface of activated monocytes as a direct result of monocyte
purification by adhesion to plastic (41), the same method used
by Kharbada et al. (40). It is well established that monocyte
purification by counterflow centrifugal elutriation does not re-
sult in the activation of these cells as assessed by the absence
of interleukin-2 receptor expression, a sensitive marker of
monocyte activation (42). The monocytes prepared in this lab-
atory and that of J uliano’s (33) were isolated by counterflow
centrifugal elutriation, so it is therefore not surprising that
both studies reach the same conclusion that monocytes/macro-
phages do not express FAK.

This study provides direct evidence for integrin-dependent
localization of paxillin to focal contacts in the absence of FAK,
and is consistent with a recent report demonstrating focal
contact formation in cells derived from FAK-deficient mice (43).
Our observations expand the implications of recent findings
that the process of tyrosine phosphorylation of paxillin by FAK
is not a critical determinant in the localization of paxillin to
focal contacts (20), and that paxillin can undergo tyrosine phos-
phorylation in the absence of FAK (43). Although the underly-
ing molecular mechanism responsible for mediating paxillin
localization to focal contacts is currently open to speculation,
Schaller et al. (44) recently established that paxillin is able to
bind specifically to synthetic peptides that mimic integrin β
subunit cytoplasmic domains. Whether paxillin binding is a
direct or indirect interaction is presently unresolved, although
it is apparent from these studies that the interaction occurs
independent of FAK (44). Our own observations, coupled with
those of other laboratories raise the distinct possibility that
upon adhesion of M-CSF-treated macrophages to vitronectin,
the localization of paxillin to focal contacts may occur via
interaction with the integrin β3 subunit cytoplasmic domain.

One of the major observations communicated in this report is
the demonstration by phosphoamino acid analysis that paxillin is
99% phosphorylated on serine residue(s) in response to α5β3-
mediated adhesion to vitronectin. While much attention in the
literature has focused on the phosphorylation of paxillin on
tyrosine residues, serine phosphorylation of paxillin in re-

Discussion

We previously reported that the colony-stimulating factor
M-CSF is able to induce expression of the αvβ3 integrin recep-
tor on the surface of cultured human macrophages (28). More-
over, we found that in M-CSF-treated macrophages adherent to
vitronectin, αv staining was localized to focal contacts. How-
ever, the identity of the β5 subunit associating with αv in focal
contacts could not be demonstrated definitively (28). Here, we
confirm by using mAbs to the β5 subunit and αvβ3 complex that
M-CSF-treated macrophages express αv only in association with
β5, and extend these observations to demonstrate that

Fig. 8. Effects of PMA and chelerythrine chloride on paxillin
localization to focal contacts. M-CSF-treated macrophages were
allowed to attach and spread on vitronectin-coated coverslips under
serum-free conditions for 60 min in the absence (A) or presence (B) of 5
ng/ml PMA. Cells were fixed, permeabilized, and stained with mAb to
paxillin. Focal contacts are highlighted by arrowheads. In panel C, cells
were pretreated with 1 μM chelerythrine chloride for 60 min prior to
attachment and spreading on vitronectin for an additional 60 min in the
absence of PMA. Cells were then fixed, permeabilized, and stained with
mAb to paxillin. Cells pretreated with GF109203X revealed a staining
pattern that was virtually identical to that seen in panel C for chel-
erythrine chloride. Bar = 10 μm.

Fig. 9. Effect of PKC inhibitors GF109203X and chelerythrine
chloride on paxillin phosphorylation. M-CSF-treated macrophages
were labeled with [32P]orthophosphate and pretreated with either di-
methyl sulfoxide (vehicle control) (lanes 1, 2, and 4), 10 μM GF109203X
(lane 3), or 1 μM chelerythrine chloride (lane 5) for 60 min as described
under “Experimental Procedures.” Cells were then maintained either in
a nonadherent state for 60 min (lane 1), or allowed to attach and spread
on vitronectin-coated Petri dishes for 60 min (lanes 2–5). To assess the
phosphorylation status of both paxillin and Src, cells were solubilized in
RIPA lysis buffer and equal concentrations and volumes of cell lysates
were immunoprecipitated using either an anti-paxillin mAb (paxillin) or
a polyclonal antiserum raised against c-src (src). The precipitated immu-
no complexes were visualized by autoradiography after SDS-PAGE.
response to integrin-mediated cell adhesion to the ECM was previously overlooked, despite the knowledge that paxillin contains multiple consensus target sites for a number of serine/threonine kinases, including PKC (18), CAMP-dependent protein kinase, casein kinase II, p34cdc2, and S6 kinase. Our observations indicate that in M-CSF-treated macrophages adherent to vitronectin, the serine phosphorylation of paxillin is mediated via a PKC-dependent mechanism. We provide evidence to show that direct stimulation of PKC activity with the phorbol ester PMA enhances paxillin phosphorylation, whereas two selective inhibitors of PKC, GF109203X and chelerythrine chloride, specifically and effectively block the phosphorylation of paxillin induced in response to vitronectin adhesion. Whether the phosphorylation of paxillin on serine is directly mediated by PKC, or alternatively, whether PKC acts indirectly to modulate the activity of other serine/threonine kinase(s) that phosphorylate paxillin is yet to be determined. Inhibition of paxillin serine phosphorylation was found to correlate with inhibition of focal contact formation in M-CSF-treated macrophages adherent to vitronectin. PKC-mediated phosphorylation changes may be an important mechanism in the assembly of focal contacts (23). The localization of PKC to focal contacts (21, 22) supports this potential role of PKC and therefore serine/threonine phosphorylation in focal contact assembly and cell spreading.

In addition to serine phosphorylation, we also demonstrate the phosphorylation of paxillin on tyrosine residue(s), albeit at very low levels, in response to αβ2-mediated adhesion of macrophages to vitronectin. In the absence of FAK expression, these data suggest that paxillin serves as a substrate for a protein-tyrosine kinase other than FAK. One obvious candidate to fulfill this role is the cytoplasmic protein-tyrosine kinase other than FAK. One obvious candidate for this role is the cytoplasmic protein-tyrosine kinase other than FAK, which was recently shown to phosphorylate paxillin on tyrosine in vivo at sites that become phosphorylated in vivo (19). Our own observations demonstrate concomitant phosphorylation of both Src and paxillin in response to integrin-mediated adhesion of M-CSF-treated macrophages to vitronectin, suggesting that paxillin tyrosine phosphorylation is coupled to Src kinase activity. The precise role of integrin-mediated tyrosine phosphorylation of paxillin remains unknown. Recent evidence indicates that tyrosine phosphorylation of paxillin is not essential for the localization of paxillin to focal contacts (20).

Our findings that paxillin localizes to focal contacts in the absence of FAK expression and is predominantly phosphorylated on serine residue(s) in response to vitronectin adhesion are intriguing, particularly in light of the recent report that tyrosine phosphorylation of paxillin is not essential for its recruitment to focal contacts (20). Whether serine phosphorylation of paxillin alone is the critical determinant in its localization to focal contacts remains to be determined, but the 99:1 predominance of serine to tyrosine phosphorylation suggests a significant functional role for this major extracellular substrate-dependent modification.

Acknowledgments—We extend many thanks to Dr. Susan Leitman and the staff of the Department of Transfusion Medicine at the National Institutes of Health for their excellent preparation of leukapheresis packs. We also thank Dr. Larry M. Wahl, Laboratory of Immunology, NIDR at NIH, for use of the elutriation system and for his valuable advice on monocyte preparation.

REFERENCES
1. Dansky, C. H., and Werb, Z. (1992) Curr. Opin. Cell Biol. 4, 772–781
2. Hynes, R. O. (1992) Cell 69, 11–25
3. Juliano, R. L., and Haskill, S. (1993) J. Cell Biol. 120, 577–585
4. Yamada, K. M., and Miyamoto, S. (1995) Curr. Opin. Cell Biol. 7, 681–689
5. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) Annu. Rev. Cell Biol. 11, 549–599
6. Turner, C. E., and Burridge, K. (1991) Curr. Opin. Cell Biol. 3, 849–853
7. Schaller, M. D., Borgmire, S. R., Vine, R. R., Reynolds, A. B., and Parsons, J. T. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 5192–5196
8. Schaller, M. D., and Parsons, J. T. (1994) Curr. Opin. Cell Biol. 6, 705–710
9. Guan, J.-L., Trethewick, J. E., and Hynes, R. O. (1991) Cell Regul. 2, 951–964
10. Burridge, K., Turner, C. E., and Romer, L. H. (1992) J. Cell Biol. 119, 893–903
11. Hanks, S. K., Calabia, M. B., Harper, M. C., and Patel, S. K. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 8487–8491
12. Konijn, L. J., Earp, H. S., Turner, C. E., Prokop, C., and Juliano, R. L. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 6392–6396
13. Lipfert, L., Halmovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) J. Cell Biol. 119, 905–912
14. Guan, J.-L., and Shalloway, D. (1992) Nature 358, 690–692
15. Turner, C. E., Glenney, J. R., Jr., and Burridge, K. (1990) J. Cell Biol. 111, 1059–1068
16. Turner, C. E. (1994) BioEssays 16, 47–52
17. Turner, C. E., Schaller, M. D., and Parsons, J. T. (1993) J. Cell Biol. 105, 637–645
18. Turner, C. E., and Miller, J. T. (1994) J. Cell Biol. 107, 1583–1591
19. Schaller, M. D., and Parsons, J. T. (1995) Mol. Cell. Biol. 15, 2635–2645
20. Bellis, S. L., Miller, J. T., and Turner, C. E. (1995) J. Cell Biol. 270, 17437–17441
21. Jensen, L., Leach, K., and Klauck, T. (1989) J. Cell Biol. 109, 697–704
22. Barry, S. T., and Critchley, D. R. (1994) J. Cell Sci. 107, 2033–2045
23. Vuori, K., and Ruoslahti, E. (1994) J. Biol. Chem. 269, 21459–21462
24. Winn, L. M., and Smith, P. (1991) in Current Protocols in Immunology (Colligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W., eds) pp. 7.6.1–7.6.6, John Wiley & Sons, Inc., New York
25. DeNicuilo, M. D., and Burns, G. F. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2517–2521
26. Mohamed, J. V., and Vassalli, P. (1987) Cell 48, 671–679
27. Siegel, J. N. (1991) in Current Protocols in Immunology (Colligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W., eds) pp. 11.2.1–11.2.7, John Wiley & Sons, Inc., New York
28. Yatohgo, T., Izumi, M., Kashiiwagi, H., and Hayashi, M. (1988) Cell Struct. Funct. 13, 281–292
29. Burns, G. F., Lucas, C. M., Krissansen, G. W., Werkmeister, J. A., Scanlon, D. B., Simpson, J. R., and Vadas, M. A. (1988) J. Cell Biol. 107, 1125–1230
30. Lin, T. H., Yurochko, A., Kornburg, L., Morris, J., Walker, J. J., Haskill, S., and Juliano, R. L. (1994) J. Cell Biol. 126, 1585–1593
31. Zachary, I., Sinnett-Smith, J., Turner, C. E., and Razengurt, E. (1993) J. Biol. Chem. 268, 22060–22067
32. Salgia, R., Jr., L. L., Lo, S. H., Brunhoff, K., Gao, A., Bhamra, A., Bauduin, C., Mathieson, J. J., Kalra, S. K., Murry, C., and Kulik, P. (1991) J. Biol. Chem. 266, 21459–21462
33. Tsukiyama, K., and Tsuboya, M. (1994) J. Biol. Chem. 269, 21459–21462
34. Sabe, H., Kata, A., Okada, H., Nakagawa, H., and Hanafusa, H. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 3984–3988
35. Weng, Z. J., Taylor, J. P., Brugge, J. S., and Delp-Duncan, C. (1993) J. Biol. Chem. 268, 14956–14963
36. Birge, R. B., Fajardo, J. E., Reichman, C., Shoelson, S. E., Songyang, Z., Cantley, L. C., and Hanafusa, H. (1993) Mol. Cell. Biol. 13, 4648–4656
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Mark O. De Nichilo and Kenneth M. Yamada

J. Biol. Chem. 1996, 271:11016-11022.
doi: 10.1074/jbc.271.18.11016

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