THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 35, Issue of August 29, pp. 21726–21734, 1997

Printed in U.S.A.

Dissection of Pathways Implicated in Integrin-mediated Actin Cytoskeleton Assembly

INVOLVEMENT OF PROTEIN KINASE C, RHO GTPase, AND TYROSINE PHOSPHORYLATION*

(Received for publication, April 1, 1997, and in revised form, June 3, 1997)

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A panel of antibodies to the αIIbβ3 integrin was used to promote adhesion of Chinese hamster ovary cells transfected with the αIIbβ3 fibrinogen receptor. While some αIIbβ3 antibodies were not able to induce p125 focal adhesion kinase (p125FAK) tyrosine phosphorylation, all the antibodies equally support cell adhesion but not spreading and assembly of actin stress fibers. Absence of stress fibers was also obtained by plating on antibodies directed to the hamster β1 integrin. In contrast, cells plated on matrix proteins spread organizing actin stress fibers. Treatment with phorbol esters phorbol 12-myristate 13-acetate (PMA) induced cells to spread on antibodies-coated dishes but not to organize actin in stress fibers. The combination of PMA and *c*ytotoxic *n*ecrotizing *f*actor 1 (CNF1), a specific Rho activator, induced cell spreading and organization of stress fibers. PMA or the combination of PMA and CNF1 also increases tyrosine phosphorylation of p125FAK in response to antibodies that were otherwise unable to trigger this response. These data show that: 1) matrix proteins and antibodies differ in their ability to induce integrin-dependent actin cytoskeleton organization (while matrix induced stress fibers formation, antibodies did not); 2) p125FAK tyrosine phosphorylation is insufficient *per se* to trigger actin stress fibers formation since antibodies that activate p125FAK tyrosine phosphorylation did not lead to actin stress fibers assembly; and 3) the inability of anti-integrin antibodies to trigger stress fibers organization is overcome by concomitant activation of the protein kinase C (PKC) and Rho pathways; PKC activation leads to cell spreading and Rho activation is required to organize actin stress fibers.

Integrins are transmembrane adhesive receptors formed by α and β subunits, which connect the matrix to the cytoskeletal structure of the cell (1). Cell matrix adhesion triggers the reorganization of cell shape and also determines important cellular functions as cell cycle entry and differentiation (reviewed in Refs. 2 and 3). Integrins trigger cytoplasmic signals such as pH variations, Ca^{2+} influx, potassium channels activation (4–6), and tyrosine phosphorylation of cytoplasmic proteins (for review, see Ref. 7). Tyrosine phosphorylation of the p125 focal adhesion kinase (p125FAK),[1] which is specifically localized in the focal contacts (8, 9), is an early event in integrin signaling in cells plated on matrix proteins (10–13) and by clustering of β1 integrins on cells adherent to plastic dishes (14) or on cells in suspension (12).

Integrin-mediated adhesion induces cytoskeletal organization, leading to actin stress fibers formation (15). The relevance of tyrosine phosphorylation to the assembly of focal adhesion and actin stress fibers has been determined by using phosphotyrosine kinase inhibitors. Herbimycin A and genistein, two known inhibitors of kinase activity, prevent focal adhesion formation and actin stress fibers assembly in cells plated on fibronectin (10, 12). Moreover treatment of cells with phosphotyrosine phosphatase inhibitors leads to increased integrin-dependent organization of focal adhesion and actin stress fibers (16). In addition to tyrosine kinases, small GTPases have been involved in the assembly of the actin cytoskeleton in response to neuropeptides and growth factors (17). In the Rho-dependent pathway, the tyrosine kinases inhibitor genistein blocks the assembly of stress fibers, suggesting a role for tyrosine kinases downstream to Rho (18). Several tyrosine kinases located in the focal adhesions, including Src and p125FAK, are potential candidates (7, 15). The exact role of p125FAK tyrosine phosphorylation in the assembly of actin stress fibers induced by the Rho pathway remains to be determined.

To assess the ability of integrins to regulate cell spreading and actin cytoskeleton organization, Chinese hamster ovary (CHO) cells transfected with the human αIIbβ3 receptor were plated on anti-human αIIbβ3 or anti-hamster α5β1 antibodies, and the roles of p125FAK kinase, protein kinase C and small GTPase Rho were analyzed. We report here that antibodies directed either to the αIIbβ3 or α5β1 trigger the organization of actin in filapodia-like structures but do not lead to actin stress fibers formation. The inability to organize stress fibers on anti-integrin antibodies is overcome by concomitant activation of PKC and Rho. Since some of the antibodies induce p125FAK tyrosine phosphorylation while others are ineffective, our data

* This work was supported by grants from the National Research Council "Progetto Finalizzato ACRO 9302149PF39"; from the Italian Association for Cancer Research (AIRC), and by Istituto Superiore di Sanita', Progetto "Sostituzioni funzionali, organi artificiali e trapianti di organi". The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: p125FAK, p125 focal adhesion kinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; CNF1, cytotoxic necrotizing factor 1; PTK, protein tyrosine kinase; CHO, Chinese hamster ovary; mAb, monoclonal antibody; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis.

also show that p125FAK tyrosine phosphorylation is not sufficient for stress fibers organization.

MATERIALS AND METHODS

Cells and Antibodies—Full-length αIIb cDNA from nucleotide 1 to 3198 was ligated into the *Sma*I pECE restriction site (19), whereas β3 cDNA from nucleotide 18 to 2585 was introduced between the *Eco*RI and *Xba*I pECE restriction sites (19, 20). The resulting plasmids were electroporated in CHO cells and clones, selected in the presence of G418 (Life Technologies, Inc., Gaithersburg, MD), were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum (Hyclone). Before each experiment, to eliminate the contribution of protein synthesis and secretion, cells were pretreated 2 h with 20 μM cycloheximide (Sigma), and adhesive assays were all performed in serum-free medium in the presence of 20 μM cycloheximide and 1 μM monensin (Sigma) (21).

Table I presents a summary of the monoclonal antibodies (mAbs) used (21–27). All the antibodies were affinity purified on protein A-Sepharose (Pharmacia Biotechnology, Uppsala, Sweden) as described (28), and the purity of the antibodies was higher than 95%.

Flow Cytometry Analysis—Cells at confluence were detached by gentle treatment with 0.25% trypsin in PBS, washed, and incubated with the indicated concentration of antibodies for 1 h at 4 °C. Cells were then incubated with 10 μg/ml affinity purified fluorescein-labeled secondary antibodies (Sigma) for 1 h at 4 °C and analyzed on the flow cytometer Facs-Star Becton Dickinson, equipped with a 5 watt argon laser at 488 nm.

Immunoprecipitation of αIIbβ3 Integrin from ^{35}S Metabolically Labeled Cells—Cells at confluence were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label, ICN Flow, Costa Mesa, CA), detergent extracted, and immunoprecipitated as described in (21). To selectively immunoprecipitate the hamster αv subunit, a polyclonal antibody prepared by injecting rabbits with a synthetic peptide reproducing the cytoplasmic domain of this subunit was used (29).

Adhesion Experiments—96-well microtiter dishes were coated with different concentrations of fibrinogen (Sigma) at 4 °C by overnight incubation and post-coated for 1 h with 1% bovine serum albumin (BSA, Sigma) in PBS. To evaluate antibody-mediated adhesion, microtiter wells were coated overnight at 4 °C with 10 μg/ml purified rabbit anti-mouse IgG (Sigma), post-coated with BSA for 1 h at 37 °C, and incubated for 2 h at 37 °C with the indicated antibodies. Cells were released from culture dishes by treatment with 5 mM EDTA for 10 min at 37 °C, washed twice with PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and plated in serum-free DMEM, 20 mM Hepes on microtiter wells for the indicated times at 37 °C. Plates were rinsed, and adherent cells were fixed with paraformaldehyde and stained with Coomassie Blue. Cell adhesion was evaluated by reading the absorbance at 540 nm in a microtiter enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad 450; Bio-Rad).

Analysis of p125FAK Tyrosine Phosphorylation by Antibody-mediated Integrin Clustering or Adhesion to Antibodies-coated Dishes—Cells at confluence were detached in 5 mM EDTA as described above, washed, resuspended in DMEM, 20 mM Hepes containing 10 μg/ml purified primary antibodies for 50 min at 4 °C, washed twice, and then incubated with 25 μg/ml purified rabbit anti-mouse immunoglobulins at 37 °C to induce integrin clustering. The cells were washed and detergent extracted in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-Cl, pH 8, 5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 trypsin inhibitory unit/ml of aprotinin) (all from Sigma) (12).

To analyze adhesion-mediated tyrosine phosphorylation, tissue culture plates of 10 cm diameter were coated either with 10 μg/ml of the indicated antibodies or with 20 μg/ml fibrinogen and postcoated with 1% BSA. As a nonspecific, substrate dishes were coated with 10 μg/ml poly-L-lysine (Sigma). Confluent cells were detached as described above and plated in DMEM, 20 mM Hepes. The cells were incubated for 1 h at 37 °C and then washed and detergent extracted as described above.

Detection of Tyrosine Phosphorylated p125FAK and of p125FAK Protein—Samples containing equal amounts of proteins were immunoprecipitated with polyclonal antibody to p125FAK (FAK-4) (16) and separated by polyacrylamide gel electrophoresis in presence of SDS (SDS-PAGE) in reducing conditions. Proteins were transferred to nitrocellulose, and the blots were incubated overnight in antiphosphotyrosine mAb PY20 (0.3 μg/ml) (Transduction) followed by peroxidase-conjugate anti-mouse IgGs (Sigma) (12, 16). Phosphotyrosil-containing proteins were visualized by the chemiluminescent detection method, ECL (Amersham, UK). Exposure times were set to obtain a linear response. To detect the p125FAK protein, the anti-p125FAK mAb 9/2 (16) was used as primary antibody.

Fluorescence Microscopy—For immunofluorescence microscopy, acid-washed glass coverslips were coated with 20 μg/ml fibrinogen or 10 μg/ml anti-integrin antibodies overnight. Cells were detached by EDTA treatment, washed, and plated for 3 h in serum-free medium with or without treatments. The cells were then fixed in 3% paraformaldehyde, 60 mM sucrose in PBS for 10 min, and permeabilized for 1 min at 4 °C in Tris-buffered saline containing 0.5% Triton X-100. Actin cytoskeleton was visualized with fluorescein-labeled phalloidin (Sigma). The coverslips were mounted in Mowiol (Aldrich) and viewed on an Olympus BH2-RFCA fluorescence microscope. Micrographs were taken on Kodak 400 film.

PMA, RO 31–8220, CNF1, Calphostin C, and Genistein Treatments—Phorbol 12-myristate 13-acetate (PMA) (Sigma) was dissolved at 10 mM in DMSO. PKC inhibitor mesylate salt RO 31–8220 (33), a gift of Dr. Hallam (Roche, Hertfordshire, UK), was prepared at 0.5 mM in DMSO and was used at final concentration of 10 μM. Calphostin C (Calbiochem, La Jolla, CA) was solubilized in DMSO at 0.2 mM and used at 100 μM. The treatments with PMA, RO 31–8220, or calphostin C were performed in serum-free DMEM supplemented with 20 mM Hepes, pH 7.4, in the presence of 20 μM cycloheximide and 1 μM monensin for the indicated times. Cytotoxic necrotizing factor 1 (CNF1) was purified as described in (30) and treatments were performed overnight in culture medium. CNF1 was also added during adhesive assays in serum-free DMEM as indicated above. Genistein (Sigma) was dissolved at 25 mg/ml in DMSO and used at the final concentration of 74 μM.

RESULTS

Expression of the αIIbβ3 Integrin in CHO Cells Confers Ability to Adhere to Fibrinogen—Fluorescence-activated cell sorter analysis showed that the transfected CHO cells express on their surface high and comparable levels of both αIIb and β3 subunits (Fig. 1*A*, *panels b-d*). Metabolically labeled cell extracts immunoprecipitated with antibodies to the human β3 subunit or to the αIIbβ3 complex indicated that the human β3 was mostly associate with the αIIb (Fig. 1*B*, *lanes a* and *c*). This was also confirmed by the fact that the anti-αv antibody, which reacts against the hamster subunit, immunoprecipitates the αvβ1 integrin, suggesting that the endogenous αv subunit preferentially forms complexes with this β subunit (Fig. 1*B*, *lane b*). While non-transfected wild-type CHO cells did not adhere to fibrinogen, transfected cells start to adhere to dishes coated with 0.625 μg/ml fibrinogen, reaching the plateau at 2.5–5 μg/ml (Fig. 1*C*). A time course experiment showed that maximal adhesion was obtained 60 min after plating (not shown).

αIIbβ3 Integrin Antibodies Have Distinct Abilities to Trigger Tyrosine Phosphorylation of the p125FAK Following αIIbβ3 Integrin Clustering on Suspended Cells—To investigate the ability of the αIIbβ3 receptor to trigger p125FAK tyrosine phosphorylation, transfected CHO cells kept in suspension were incubated with monoclonal antibodies directed to different epitopes of the αIIb, β3 subunits, or the αIIbβ3 complex, and clustering was induced by the addition of a secondary antibody (see Table I for summary of the antibodies) (21–27). Clustering by anti-β3 mAb P37 induced high levels of p125FAK tyrosine phosphorylation, indicating that integrin aggregation by antibodies was sufficient to trigger this event. Maximal stimulation of p125FAK tyrosine phosphorylation was obtained as early as 2 min after clustering, persisted at 10 min, and decreased at 30 min of incubation (Fig. 2*A*).

The different antibodies used showed distinct abilities to trigger p125FAK tyrosine phosphorylation. In addition to mAb P37, anti-αIIbβ3 mAbs P4.1.1 and P8.2.1 and anti-β3 mAb B212 were all capable of inducing p125FAK tyrosine phosphorylation (see Table I). In contrast, we found that three antibodies, D33C directed to the αIIb subunit (Fig. 2*B*) (22), CS3, and P9.1.1 (not shown) to the αIIbβ3 complex, were completely unable to induce p125FAK tyrosine phosphorylation. The lack of p125FAK tyrosine phosphorylation observed with these antibodies was reproducibily found in five different experiments (see also the adhesion experiments presented below).

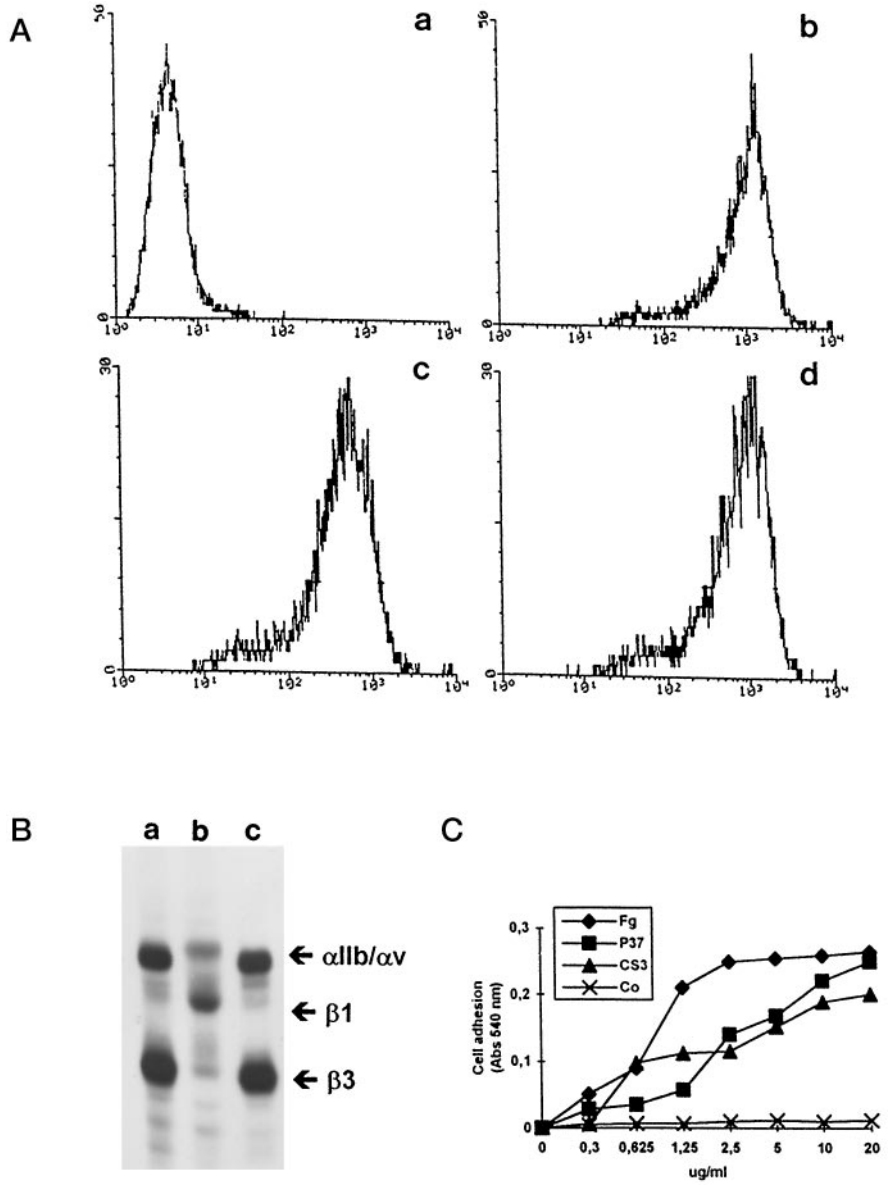

FIG. 1. **Expression of human αIIbβ3 integrin transfected in CHO cells and adhesion of transfected cells to fibrinogen.** *A*, flow cytometry analysis. Nontransfected CHO cells (*panel a*) and cells transfected with the αIIbβ3 (*panels b-d*) were incubated in suspension with 10 μg/ml mAb B212 to the β3 subunit (*panels a* and *b*), mAb D33C to the αIIb subunit (*panel c*), or mAb CS3 to the complex αIIbβ3 (*panel d*) for 1 h at 4 °C, followed by fluorescein-labeled secondary antibodies. The data are expressed as cell number (*Y axis*) plotted as a function of fluorescence intensity (*X axis*) and are representative of three separate experiments. *B*, immunoprecipitation of the human αIIbβ3 integrin. Transfected CHO cells metabolically labeled with [^{35}S]methionine were immunoprecipitated with mAb B212 (*lane a*), a polyclonal antibody to the αv subunit (*lane b*), or mAb CS3 (*lane c*). Radioactive antigens were separated by 6% SDS-PAGE in non-reducing conditions and visualized by fluorography after 3 days of exposure at −80 °C. The positions of the α and β subunits are indicated. *C*, cells suspended with EDTA treatment were plated in serum-free medium at 37 °C on 96-well microtiters coated with increasing concentrations of fibrinogen, mAb P37, or mAb CS3 for 1 h. Adherent cells were washed with PBS, fixed, and stained with Coomassie Blue. *Y axis* represents absorbance.

The ability of different antibodies to trigger p125FAK tyrosine phosphorylation may be due to the binding properties of the antibodies. To evaluate if the antibodies used differ in their capacity to induce αIIbβ3 clustering in the plasma membrane, immunofluorescence experiments were performed. When cells were stimulated with mAbs P37 or D33C and secondary antibodies at 4 °C, the αIIbβ3 protein appeared uniformly diffused on the cell surface (Fig. 3, panel *a*). When the temperature was raised to 37 °C, the antibodies gave rise to redistribution of the antigen on the cell surface and formation of patches (Fig. 3, *panels e-n*), indicating that both mAb P37 and D33C were able to cluster the molecules on the plasma membrane. Staining with phalloidin showed that actin cytoskeleton reorganizes under the clustered receptors (not shown). Similar integrin redistribution and actin organization was found with the different antibodies used (Table I). Interestingly, cluster formation and actin reorganization were independent from the ability of the different antibodies to trigger p125FAK tyrosine phosphorylation since mAbs D33C, CS3, and P9.1.1 induced actin co-clustering without triggering p125FAK tyrosine phosphorylation.

p125FAK Tyrosine Phosphorylation Is Induced by Adhesion to Fibrinogen or αIIbβ3 Integrin Antibodies—To test if lack of p125FAK tyrosine phosphorylation observed in clustering experiments in suspended cells was related to the absence of cell spreading, cells were plated for 1 h on anti-αIIbβ3 antibodies-coated tissue culture dishes. The ability of the anti-αIIbβ3 antibodies to trigger p125FAK tyrosine phosphorylation was very similar to that observed in clustering experiments (Fig. 4; see also Table I). While cells plated on mAb P37 and mAbs P4.1.1 or P8.2.1 were able to induce a level of p125FAK tyrosine phosphorylation comparable with that observed on fibrinogen, the αIIbβ3-specific ligand used as positive control (not shown), mAbs D33C and CS3 were poorly effective. The inability of the latter two antibodies to trigger p125FAK tyrosine phosphorylation was also found when plating of cells was protracted for 3 h (not shown). Adhesion experiments (Fig. 1*C*) showed that transfected cells adhere in a similar way either to mAb CS3 (ineffective in the induction of p125FAK tyrosine phosphorylation) or to mAb P37 (effective in the induction of p125FAK tyrosine phosphorylation), indicating that the two antibodies were equally capable of promoting cell attachment. These data show that the epitopes recognized by mAbs D33C and CS3 are able to trigger comparable levels of cell adhesion but are ineffective in stimulating tyrosine phosphorylation of p125FAK kinase both in suspended and in adherent cells. In

TABLE I

Properties of anti-integrin antibodies and ability to trigger p125FAK tyrosine phosphorylation

The table summarizes the properties of the monoclonal antibodies used in this study and their ability to trigger p125FAK tyrosine phosphorylation. Binding specificities for α and β subunits or integrin complexes are indicated. The ability to induce p125FAK tyrosine phosphorylation is reported both in adhesion experiments and in clustering in nonadherent cells. + indicates levels of p125FAK tyrosine phosphorylation comparable to that observed on fibrinogen, used as positive control (not shown). Fluorescence analysis in clustering indicates the ability of the monoclonal antibodies to aggregate the integrin molecules on the plasma membrane, giving rise to a patch redistribution of the antigen on the cell surface (see also Fig. 3). ND, not determined.

Antibodies	Binding specificity	Adhesion: FAK tyrosine phosphorylation	Clustering: FAK tyrosine phosphorylation	Clustering: fluorescence analysis
D33C	IgG1 anti-αIIb, induces fibrinogen binding and platelets aggregation (22)	−	−	+
P37	IgG1 anti-β3, binds to unstimulated platelets with no functional effects (27)	+	+	+
B212	IgG1 anti-β3, blocks interaction with adhesive proteins (21)	+	+	+
CS3	IgG1 anti-αIIbβ3, induces fibrinogen binding and platelets aggregation (22)	−	−	+
P 9.1.1	IgG2b anti-αIIbβ3, binds to both unstimulated and thrombin-stimulated platelets, blocks fibrinogen binding and platelets aggregation (24)	+	−	+
P 4.1.1	IgG1 anti-αIIbβ3, partially inhibits fibrinogen binding, binds to both unstimulated and thrombin-stimulated platelets (25)	+	+	+
CP 8.2.1	IgG1 anti-αIIbβ3, binds to both unstimulated and thrombin-stimulated platelets, blocks fibrinogen binding (24)	+	+	+
PB1	IgG1 anti-hamster α5β1, inhibits binding to fibronectin (26)	+	ND	ND
7E2	IgG anti-hamster β1, no functional effects (23)	+	+	+

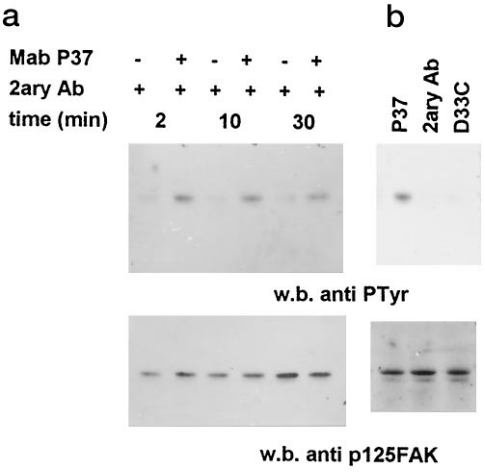

FIG. 2. **Clustering of suspended cells with αIIbβ3 integrin antibodies is able to trigger p125FAK tyrosine phosphorylation.** *A*, cells were brought in suspension by brief trypsin treatment, incubated 50 min at 4 °C with 10 μg/ml mAb P37 to human β3, and further incubated with 25 μg/ml purified rabbit anti-mouse immunoglobulins for 2, 10, or 30 min at 37 °C to induce integrin clustering. *B*, cells in suspension were incubated 50 min at 4 °C with 10 μg/ml mAb P37 or mAb D33C and further incubated with 25 μg/ml purified rabbit anti-mouse immunoglobulins for 10 min at 37 °C to induce integrin clustering. *2ary Ab* means that this sample was not incubated with the primary antibody. p125FAK immunoprecipitated with FAK4 polyclonal antibody was separated on 6% SDS-PAGE and visualized by Western blotting with mAb PY20 (*top*) or anti-p125FAK mAb 9/2 (*bottom*). *w.b.*, Western blot.

contrast, mAb P9.1.1, which was unable to trigger tyrosine phosphorylation of p125FAK in suspension, was effective in adhesion experiments, indicating that the binding site for this antibody elicits a different response during cell adhesion or integrin clustering in suspension.

Cell Adhesion to Fibrinogen or to Integrin Antibodies Differently Support Actin Organization—Since p125FAK tyrosine phosphorylation has been related to the ability of cells to organize focal adhesions and actin stress fibers, actin cytoskeleton was analyzed in cells plated for 3 h on αIIbβ3 antibodies-coated dishes. Cells plated on mAbs P37, P9.1.1, D33C, and CS3 attached and spread only partially, but in no case did they organize actin cytoskeleton in stress fibers (Fig. 5, *panels b-e*). A similar pattern of actin organization was found in cells plated on 10 or 100 μg/ml antibodies, indicating that inability to trigger cell spreading and actin stress fibers organization was not dependent on ligand density. Cell adhesion to a 1:1 mixture of the different antibodies was also unable to induce organization of actin stress fibers (not shown). Similar organization of actin cytoskeleton was obtained by plating cells on mAb 7E2 to hamster β1 subunit or mAb PB1 to the hamster α5β1 complex (23, 26) (see Figs. 6 and 7). In cells plated on antibodies, actin was distributed on the periphery of the cells forming filopodia-like structures and large membrane extensions similar to lamellipodia (see for example, Fig. 5, *panel b*, and Fig. 6, *panel b*). In contrast, the cells spread completely on fibrinogen, and actin was organized in stress fibers (Fig. 5, *panel a*). Treatment of cells with cytochalasin D, an inhibitor of actin polymerization, completely abolished actin organization, either in filopodia- or lamellipodia-like structures on monoclonal antibodies (data not shown). These data show that antibody occupation of specific epitopes of the αIIbβ3 integrin is able to induce actin filopodia- and lamellipodia-like structures but not full cell spreading nor stress fibers, as seen when cells bind to purified fibrinogen. Formation of filopodia- and lamellipodia-like structures was observed both on anti-αIIbβ3 antibodies able to trigger p125FAK tyrosine phosphorylation (P37, P9.1.1., P4.1.1, P8.2.1, mAb B212) than on antibodies incapable of inducing p125FAK tyrosine phosphorylation (mAbs D33C and CS3), indicating that phosphorylation of p125FAK was not necessary to the actin organization reached by cells plated on anti-αIIbβ3 antibodies. Moreover, since plating cells on anti-αIIbβ3 mAbs able to trigger p125FAK tyrosine phosphorylation did not lead to the actin stress fibers organization seen on fibrinogen, these data also indicate that p125FAK phosphorylation is not sufficient to the assembly of actin stress fibers.

Cell Spreading and Actin Stress Fibers Formation in Cells Plated on Anti-integrin Antibodies: Involvment of PKC and Rho GTPase—Protein kinase C has been implicated in the ability of cells to spread and to organize focal adhesions on matrix proteins (31, 32). To test whether the inability of cells plated on anti-integrin antibodies to spread completely and to organize actin stress fibers could be overcome by activation of the PKC pathway, cells were plated on mAb B212 to the β3 subunit or mAb 7E2 to the hamster β1 subunit in the presence of PMA, a known activator of PKC. PMA-treated cells appeared well spread within 1 h of adhesion (Fig. 6, *panels c* and *d*), whereas nontreated cells remain roughly round or only partially spread (Fig. 6, *panels a* and *b*). PMA induced spreading at 50 nM, and maximal extent of cells spreading (evaluated as 90% of the cells becoming spread) was obtained at 100 nM. PMA-treated cells acquired a very flat morphology, and the majority of the cell

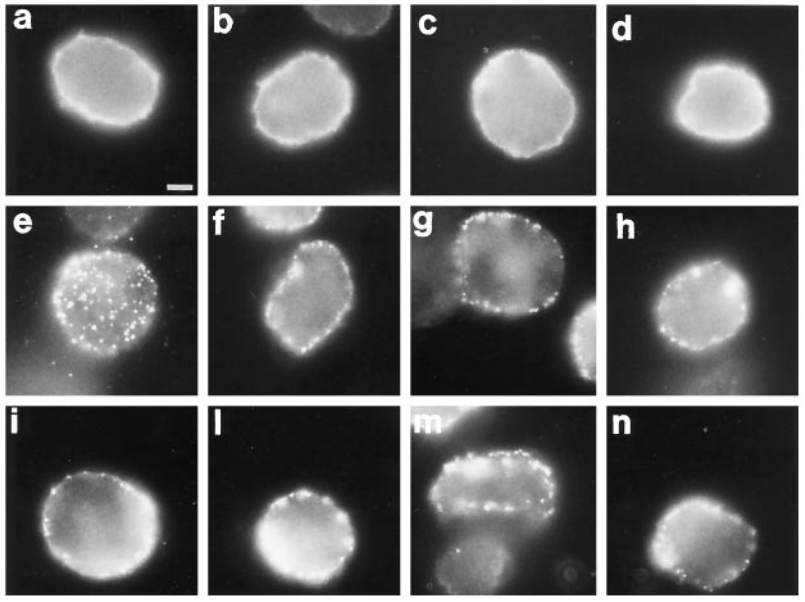

FIG. 3. **Immunofluorescence analysis of αIIbβ3 and actin in clustered cells.** Cells were brought in suspension by brief trypsin treatment, incubated 50 min at 4 °C with 10 μg/ml mAb D33C (*panels a, b,* and *e-h*) or 10 μg/ml mAb P37 (*panels c, d,* and *i-n*), and further incubated with 25 μg/ml purified rabbit anti-mouse immunoglobulins for 10 min at 4 °C (*panels a-d*) or at 37 °C (*panels e-n*). Cells were fixed, permeabilized, and stained with rhodamine-labeled anti-mouse immunoglobulins. In *panel e*, a representative view of the integrin patches at the top of the cells is shown. *Scale bar*, 5 μM.

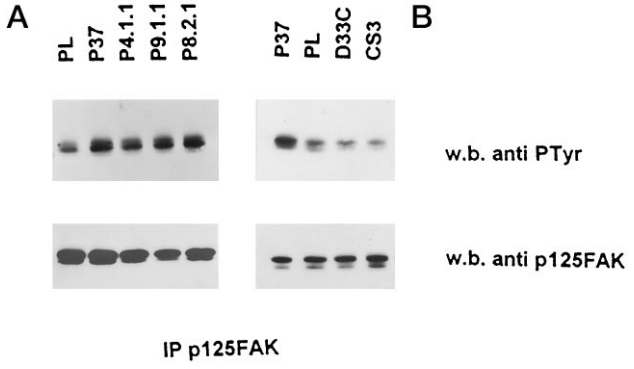

FIG. 4. **p125FAK tyrosine phosphorylation following adhesion of αIIbβ3-transfected CHO cells to antibodies-coated dishes.** Cells were detached and plated for 1 h on culture dishes coated with 10 μg/ml poly-lysine (*PL*) or 10 μg/ml of the indicated mAbs. *A*, immunoprecipitated p125FAK was visualized by Western blotting with mAb PY20 (*top*) or anti-p125FAK mAb 9/2 (*bottom*). Gels shown in *panels A* and *B* were from two separate experiments. *w.b.*, Western blot.

population lost the filopodia-like extensions that characterized the untreated cells. At higher PMA concentrations, 500 nM, however, the peripheral cell edges start to detach from the substratum, suggesting formation of membrane ruffling (not shown). PMA treatment only promoted formation of small bundles of actin filaments in 30–50% of the cells, predominantly located at the periphery of the cell. Otherwise actin remained organized in small patches in the cytoplasm or at the boundary of the cell (Fig. 6, *panels c* and *d*). To further investigate whether the effects observed with PMA were indeed due to PKC activation, cells were plated on mAb B212 in the presence of a selective PKC inhibitor, the mesylate salt RO 31–8220 (33). RO 31–8220-treated cells in the presence of PMA remained round and poorly attached, showing that the inhibitor prevented PMA-induced full spreading (Fig. 6, *panel e*). Moreover, cells also treated with RO 31–8220 and plated on anti-αIIbβ3 antibodies in the absence of PMA remained round (not shown). RO 31–8220 treatment also abolishes cell spreading on matrix proteins, fibrinogen, or vitronectin (not shown). Similar results were obtained by treating cells with calphostin C, another inhibitor of PKC (not shown), suggesting that basal activation of PKC may occur in cells adherent to antibodies-coated dishes and may be involved in organization of filopodia- and lamellipodia-like structures.

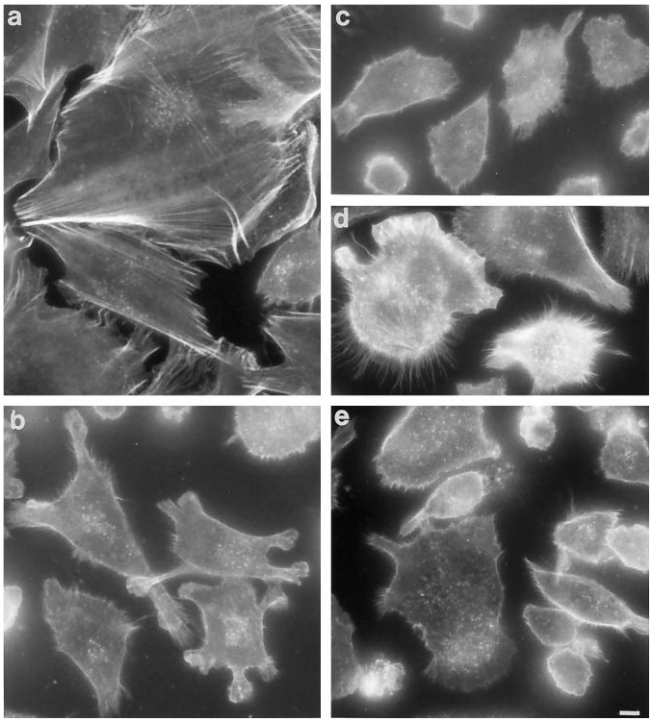

FIG. 5. **Actin cytoskeletal organization of transfected CHO cells plated on fibrinogen or monoclonal antibodies to the αIIbβ3 integrin.** Cells were gently detached with EDTA and plated for 3 h on acid-washed glass coverslips coated with 20 μg/ml fibrinogen (*panel a*) or 10 μg/ml mAb D33C (*panel b*), mAb P37 (*panel c*), mAb CS3 (*panel d*), or mAb P9.1.1 (*panel e*). Cells were fixed, permeabilized, and stained with phalloidin for F-actin. *Scale bar*, 5 μM.

Previous work showed that genistein-sensitive tyrosine kinases are implicated in integrin-mediated cell adhesion (12). Cells plated on anti-integrin antibodies in the presence of PMA and genistein did not spread completely (Fig. 6, *panel f*), indicating that tyrosine kinase activity is implicated in PMA-induced cell spreading.

Since PMA-mediated PKC activation did not appear to be sufficient for full organization of actin stress fibers, it is possible that this event requires the small GTPase Rho (17, 34, 35). To investigate a role for Rho in actin cytoskeleton organization following integrin antibody binding, we used the bacterial toxin

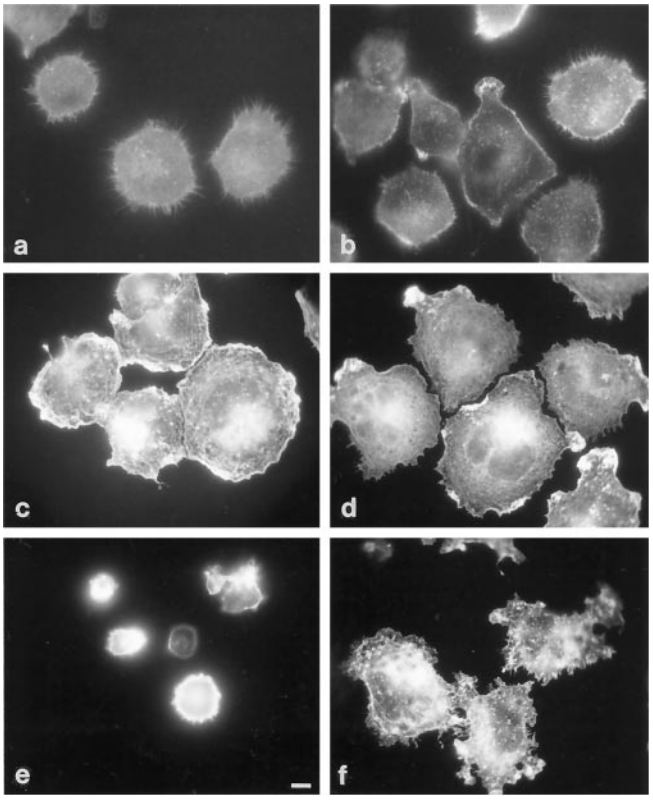

FIG. 6. **PMA treatment allows cells to spread on monoclonal antibodies to the αIIbβ3 integrin or the hamster β1 subunit.** Cells were brought in suspension by EDTA treatment and plated 1 h on anti-β3 mAb B212 (*panels a, c, e*, and *f*) or on anti-hamster β1 mAb 7E2 (*panels b* and *d*). Cells were treated in *panels c, d*, and *f*, with 100 nM PMA; in *panel e*, with PMA in the presence of 10 μM RO 31–8220, and in *panel f*, with PMA in the presence of 74 μM genistein. Cells were fixed, permeabilized, and stained with phalloidin for F-actin. *Scale bar*, 5 μM.

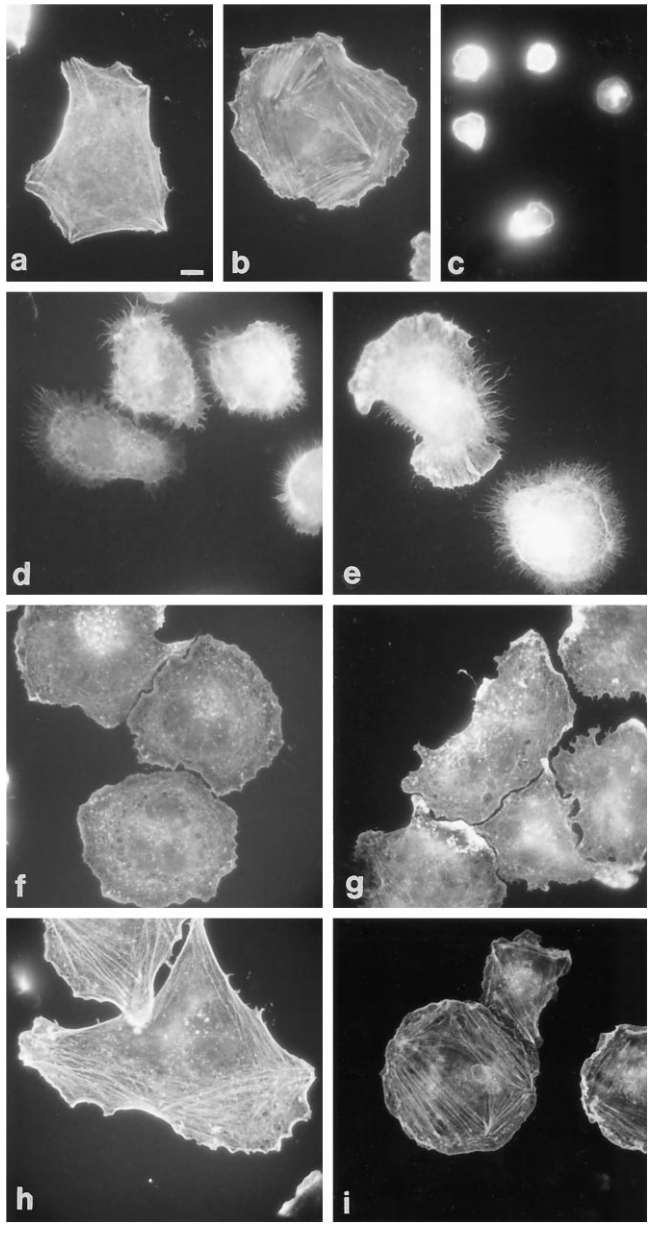

FIG. 7. **CNF1 treatment leads to stress fibers assembly.** Cells brought in suspension by EDTA treatment were plated for 1 h on vitronectin (*panels a* and *b*), anti-αIIbβ3 mAb P4.1.1 (*panels c, d, f*, and *h*), or anti-α5β1 mAb PB1 (*panels e, g*, and *i*). *Panels a, d*, and *e*, untreated cells; *panels b* and *c*, 10^{-6} M CNF1; *panels f,* and *g*, 100 nM PMA; *panels h,* and *i*, 10^{-6} M CNF1 and 100 nM PMA. Cells in *panels b, c, h*, and *i* were pretreated with CNF1 by overnight incubation. Cells were fixed, permeabilized, and stained with phalloidin for F-actin. *Scale bar*, 5 μM.

Escherichia coli CNF1, known to activate Rho (26, 36–38). As preliminary experiments, transfected cells were treated with 10^{-6} M CNF1 and plated on vitronectin. CNF1 treatment increases the number of stress fibers in 100% of the cells, showing a more densely packed organization of stress fibers than in untreated cells (Fig. 7, *panels a* and *b*).

To investigate whether CNF1-mediated constitutive activation of Rho might affect actin stress fibers formation on anti-αIIbβ3 antibodies, cells treated with 10^{-6} M CNF1 were plated on anti-αIIbβ3 antibodies. The cells attached poorly and remained completely round (Fig. 7, *panel c*). Prolonged times of adhesion (from 3 to 20 h) or lower doses of CNF1 (from 10^{-7} to 10^{-10} M) did not lead to cell spreading on antibodies and actin stress fibers organization (not shown). This rather surprising result indicates that activation of Rho *per se* does not support actin stress fibers organization when integrins are occupied by antibodies. Different results were obtained when cells were treated with the combination of PMA and CNF1. In this condition, after 1 h of adhesion, cells organize a prominent array of actin stress fibers on anti-αIIbβ3 or α5β1 integrins antibodies (Fig. 7, *panels h* and *i*), indicating that both PKC and Rho activation are necessary to reach actin stress fibers organization.

p125FAK Tyrosine Phosphorylation Is Increased by PMA and PMA/CNF1 Treatments—To test whether PKC and Rho activation might modify p125FAK tyrosine phosphorylation, PMA- or PMA and CNF1- treated cells were plated on mAb D33C, which is not able to trigger p125FAK tyrosine phosphorylation (see also Fig. 4). Cells plated on mAb D33C in the presence of PMA show induction of p125FAK tyrosine phosphorylation (Fig. 8). The relevance of tyrosine kinase activity in PMA-induced cell spreading was also shown by the fact that genistein, a known inhibitor of tyrosine kinases, blocks PMA-induced cell spreading (see Fig. 6, *panel f*). Moreover, combined treatment with CNF1 and PMA caused a slight additional increase in the level of p125FAK tyrosine phosphorylation. These data indicate that p125FAK phosphorylation can be induced by activation of PKC and Rho pathways.

DISCUSSION

In this study, we transfected the human αIIbβ3 integrin in CHO cells, and we used a panel of monoclonal antibodies directed either to the human αIIbβ3 or to the endogenous ham-

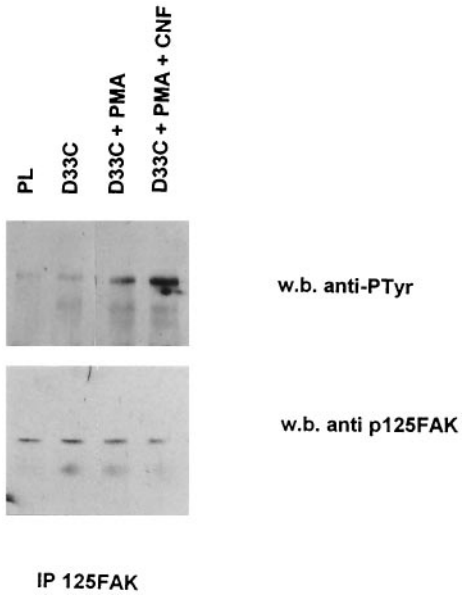

FIG. 8. **p125FAK tyrosine phosphorylation is increased by PMA or PMA and CNF1 treatment.** Cells were detached and plated for 1 h on culture dishes coated with poly-lysine (*PL*) or 10 μg/ml mAb D33C in the presence of 100 nM PMA, of 100 nM PMA and 10^{-6} M CNF1, or were left untreated. p125FAK was immunoprecipitated and visualized by Western blotting with mAb PY20 (*top*) or anti-p125FAK mAb 9/2 (*bottom*). *w.b.*, Western blot.

ster $\alpha5\beta1$ integrin complexes to dissect the signaling pathways involved in actin organization. We found that while most of the antibodies induce p125FAK tyrosine phosphorylation, some of them were ineffective. All the antibodies, however, support adhesion and partial actin organization but not full spreading and stress fibers polymerization. In contrast, cells spread and organize stress fibers on fibrinogen. We found that the combined action of PKC activator PMA and Rho activator CNF1 is required to trigger stress fibers organization following adhesion to anti-integrin antibodies. These results allow us to draw the following conclusions. 1) Different integrin ligands trigger different actin organization; matrix proteins, such as fibrinogen, are able to induce actin stress fibers formation, while antibodies to integrins induce filopodia and lamellipodia but not stress fibers. 2) Actin organization in filopodia- and lamellipodia-like structures does not require p125FAK tyrosine phosphorylation. 3) Tyrosine phosphorylation of p125FAK in response to integrin occupancy is not sufficient *per se* to lead to stress fibers organization. 4) To obtain integrin-dependent full cell spreading and actin stress fibers organization, at least two events are required, activation of PKC and Rho.

In this work, we found that the phosphorylation of p125FAK kinase can be driven only by antibody binding to specific epitopes of the αIIb$\beta3$ integrin. The antibodies described in Table I bind to distinct epitopes of the receptor, some of them (mAbs P9.1.1, P8.2.1, P4.1.1) inhibit ligand binding (24, 25), while others (D33C, CS3) induce fibrinogen binding mimicking αIIb$\beta3$ activation via ADP (22). The ability to trigger p125FAK tyrosine phosphorylation does not depend on the ability of the antibodies to block ligand binding. On the other hand, two of the antibodies that were ineffective at inducing p125FAK tyrosine phosphorylation, D33C and CS3, are activating antibodies (22). The inability of these mAbs to trigger p125FAK tyrosine phosphorylation was observed both in clustering in suspension and in adhesion to antibodies-coated dishes. Similar data were reported by Pelletier *et al.* (39). Shattil and co-workers showed that an antibody with properties comparable with D33C and CS3 induces p125FAK tyrosine phosphorylation in platelets only in the presence of a second stimulus (40). This is similar to our data showing that PMA and CNF1 are necessary to obtain p125FAK tyrosine phosphorylation in cells plated on mAb D33C. In addition, however, we show that using different antibodies, clustering of the αIIb$\beta3$ is sufficient to trigger p125FAK tyrosine phosphorylation in CHO cells expressing this receptor. Phosphorylation of the p125FAK in αIIb$\beta3$-transfected cells has also been very recently described (41). These data and the present work indicate clearly that the αIIb$\beta3$ integrin is able to trigger the cytoplasmic pathway leading to tyrosine phosphorylation of the p125FAK kinase in response to integrin ligand binding. The requirement of a costimulus in αIIb$\beta3$-induced p125FAK tyrosine phosphorylation in platelets may be due either to the properties of the specific activating antibody used or to a platelet-specific signaling mechanism.

The data presented here indicate that antibodies to either $\alpha5\beta1$ or αIIb$\beta3$ integrins are able to support cell adhesion, but they are incapable of triggering stress fibers formation. This experimental system allows us to test biological pathways implicated in integrin-mediated cells adhesion and cytoskeletal organization. Cells plated on the different antibodies were poorly spread, and F-actin was organized in small patches and in peripheral thin filopodia-like structures. Similar structures have been previously described in the early phases of cell adhesion, as a peculiar actin organization during focal adhesion formation, in a step which precedees accumulation of cytoskeletal elements (42, 43). Assembly of short bundles of actin filaments to produce microspikes and filopodia have also been previously demonstrated in motile cells and at the end of neuron growth cones (44). The formation of filopodia and lamellipodia is induced by Cdc42 and Rac, belonging to the Rho family of small GTP-binding proteins (17, 45). We can hypothesize that interaction with monoclonal antibodies leads to integrin-dependent activation of Cdc42 and Rac. Thus, cell adhesion to the monoclonal antibodies to integrin receptors leads to an initial level of actin organization, but it is insufficient to drive redistribution of cytoskeletal components to organize actin in stress fibers.

We found that concomitant treatment with PMA and CNF1, activators of PKC and Rho respectively, is required to obtain cell spreading and actin stress fibers organization in cells plated on anti-integrin antibodies. These pathways need to be activated in cells plated on antibodies directed either to $\beta1$ or $\beta3$ integrin subunits, indicating that both $\beta1$- or $\beta3$-mediated integrin clustering requires similar intracellular pathways to trigger actin stress fibers formation.

PKC and Rho appear to play different roles in controlling these processes, while PKC controls cell spreading and Rho in combination with PKC regulates organization of stress fibers. In the presence of the phorbol ester PMA, filopodia disappear, and the cells become round, well spread with large lamellipodia. Short and thin bundles of actin filaments, however, were visible, located at the periphery of the cells, and actin was mainly organized in small patches inside the cytoplasm. Since this effect was blocked by the PKC inhibitors RO 31–8220 and calphostin C, these data strongly suggest that cell spreading on integrin antibodies is regulated by PKC. PKC involvment in spreading and focal adhesion formation has been demonstrated in cells plated on matrix proteins (31, 32, 46). Using specific inhibitors or activators, it has been shown that PKC regulates spreading of platelets on fibrinogen (46) and of fibroblasts on fibronectin (32) or the ability of fibroblasts to organize focal adhesions on fibronectin (31). It has been recently reported that syndecan 4, a proteoglycan localized in the focal adhesions in a PKC dependent manner, can activate PKC and thus may

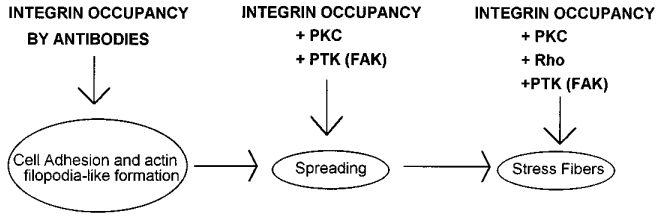

FIG. 9. **Model of integrin-mediated signaling events leading to actin cytoskeleton organization.** Integrin occupancy by specific antibodies leads to cell adhesion and actin organization in filopodia-like structures. This event does not require p125FAK tyrosine phosphorylation since antibodies that do not trigger p125FAK tyrosine phosphorylation lead to the same cytoskeletal organization. PMA induces cells spreading and stimulates p125FAK tyrosine phosphorylation following adhesion to integrin antibodies that are *per se* ineffective in inducing this event. PMA-induced spreading is also blocked by genistein. Thus, these data indicate that PKC and PTK are necessary to obtain cell spreading on anti-integrin antibodies. The combined treatment with CNF1 and PMA triggers stress fibers formation and increases p125FAK tyrosine phosphorylation, indicating that stress fibers assembly following adhesion to anti-integrin antibodies requires activation of PKC, Rho, and PTK.

function as a co-receptor with integrins, interacting with the heparin binding domain exposed on the fibronectin molecule (47).

Our results show that stress fibers formation requires additional treatment with the Rho activator CNF1. The action of CNF1 is specific for Rho since this toxin activates Rho by specifically deamidating glutamine at position 63 (38). Interestingly in our system, we can show that Rho, although essential, is not sufficient to drive actin stress fibers formation. In fact, cells treated with CNF1 remained round and did not organize stress fibers on antibodies-coated dishes. A dose-response experiment, where cells were treated with decreasing doses of CNF1, did not allow us to find a condition in which CNF1-treated cells were able to spread on anti-integrin antibodies. Since activation of Rho has been shown to stimulate cell contractility (48), it is possible that in cells where Rho has been constitutively activated by CNF1, cell contractility may antagonize cell spreading. In contrast, cells plated on vitronectin respond to CNF1 by organizing thick and prominent stress fibers, further showing that matrix proteins and integrin antibodies elicit distinct intracellular signaling responses.

The data reported in this work suggest that, in addition to PKC and Rho activation, tyrosine kinases are likely to be important in regulation of stress fibers formation. The results presented here support the idea that tyrosine phosphorylation of p125FAK *per se* is not sufficient to induce organization of stress fibers since cells adhering to antibodies able to trigger p125FAK tyrosine phosphorylation do not organize actin stress fibers. PKC and Rho activation, however, induces tyrosine phosphorylation of p125FAK kinase in cells plated on antibodies that are not able *per se* to activate this phosphorylation event. According to these data, we and others have previously shown that protein tyrosine kinase (PTK) inhibitors blocked formation of stress fibers (10, 12), thus suggesting that PTKs are required in addition to PKC and Rho to achieve organization of actin stress fibers in response to integrin ligand binding (Fig. 9). Contrasting results on the role of p125FAK tyrosine phosphorylation in stress fibers and focal adhesion assembly have been presented (49). Cells deriving from p125FAK knockout mice assemble focal adhesions in the absence of p125FAK but reduce their motility (50). Gilmore and Romer (51), using a dominant negative form of p125FAK, found a decreased cell motility but not interference on focal adhesion organization. These results suggest that p125FAK or its tyrosine phosphorylation, rather than playing a structural role, may be crucial in regulating turn-over of stress fibers and focal adhesions.

Involvment of PKC and Rho in p125FAK tyrosine phosphorylation is in agreement with findings showing that PMA can trigger p125FAK tyrosine phosphorylation in cells plated on matrix proteins (32, 46). The ability of Rho to trigger p125FAK tyrosine phosphorylation has been recently reported, following scraping of activated Rho into cells (52).

The data presented above indicate that p125FAK tyrosine phosphorylation is not able to trigger PKC or Rho activation since antibodies capable of inducing p125FAK phosphorylation did not lead to cell spreading or actin stress fibers, processes which require PKC and Rho pathways. At the same time, PKC does not trigger Rho activation and *vice versa*, as shown by the fact that stimulation by PMA only leads to cell spreading but not to stress fibers organization. CNF1-mediated Rho activation is also unable to drive actin cytoskeleton organization to stress fibers. Thus p125FAK tyrosine phosphorylation and PKC and Rho activation are three independent pathways, which are all required to trigger full cell spreading and actin stress fibers. In our system, anti-integrin antibodies are able to trigger only the pathway leading to p125FAK tyrosine phosphorylation, while fibrinogen is likely to activate all three pathways.

Our data show that organization of filopodia-like structures does not require induction of p125FAK tyrosine phosphorylation by αIIbβ3. In fact, the αIIbβ3-dependent organization of filopodia-like structures occurs also with antibodies that are incapable of activating this signaling event. Moreover, the ability of the αIIbβ3 integrin to organize F-actin independently from p125FAK tyrosine phosphorylation was demonstrated by clustering of the αIIbβ3 integrin on the surface of cells in suspension. In these cells, in fact, clustering with an antibody incapable of triggering p125FAK tyrosine phosphorylation, leads to actin co-clustering. Thus, actin aggregation under the clusterized receptor or formation of filopodia-like structures in adhesion to antibodies-coated dishes are events independent from p125FAK tyrosine phosphorylation. Whether other tyrosine kinases are involved is not known at present. Tyrosine kinase activity is required for accumulation of F-actin and other focal adhesion proteins in the clustering site (53). The data presented here indicate that p125FAK tyrosine phosphorylation is not involved in this step.

In conclusion, the data reported in this paper indicate that PKC and Rho are required in cell spreading and actin stress fibers assembly following integrin-dependent signaling. Although PKC involvment in spreading or focal adhesion formation on fibronectin has been previously shown (31, 32) and Rho activation has been demonstrated to regulate actin stress fibers formation following mitogen stimulation in mouse fibroblasts (35, 45), the data presented here are the first demonstration in a single cell line that following integrin-ligand binding, PKC and Rho are both necessary to the establishment of cell spreading and actin stress fibers organization.

Acknowledgments—We are grateful to Drs. J. Gonzales-Rodriguez and Zaverio Ruggeri for the generous gift of antibodies. We also thank Dr. S. Shattil for helpful comments on the manuscript.

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