Control of the $\alpha_5\beta_1$ Integrin/Fibronectin Interaction in Vitro by the Serine/Threonine Protein Phosphatase Calcineurin†

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ABSTRACT: Using Chinese hamster ovary cell lysate, an *in vitro* assay has been developed to study the interaction of fibronectin with the $\alpha_5\beta_1$ integrin in a cytosolic environment. In our solid phase assay, 96-well microtiter plates were coated with fibronectin in which cell lysate was incubated. A dose-dependent binding of the fibronectin receptor onto the coated plastic was immunodetected by specific polyclonal antibodies raised against the $\alpha_5\beta_1$ integrin. Both soluble fibronectin and PB1, a monoclonal antibody raised against the fibronectin receptor, competed with the $\alpha_5\beta_1$ integrin for binding to the fibronectin-coated plastic. General phosphatase inhibitors used during cell lysis completely abolished the fibronectin/integrin interaction in the assay, indicating that the affinity of the fibronectin receptor might be modulated by a protein phosphatase activity. Furthermore, in this assay, the interaction between the fibronectin receptor and its substrate in a cytosolic environment required intracellular calcium. Additionally, the action of more specific phosphatase inhibitors and the inhibition of the integrin/fibronectin interaction by a monoclonal antibody raised against the calcium/calmodulin-dependent protein phosphatase calcineurin suggested that calcineurin allowed the interaction between the $\alpha_5\beta_1$ integrin and fibronectin. Metabolical labeling experiments showed that $\alpha_5\beta_1$ itself was not the target of phosphorylation/dephosphorylation cascades involving calcineurin and leading to the modulation of integrin affinity. Taken together, these results showed that *in vitro* one substrate of the serine/threonine protein phosphatase calcineurin regulates the $\alpha_5\beta_1$ integrin affinity by interacting with a yet unidentified effector.

The integrin family is constituted of transmembrane heterodimeric ($\alpha\beta$) receptors involved in both cell−cell and cell−matrix interactions and clustered into subcellular structures named focal adhesion (Hynes, 1992). The intracellular domain of the β subunit binds talin (Horwitz et al., 1986) and α-actinin *in vitro* (Otey et al., 1990). It contains all the information required to allow the recruitment *in vivo* of the receptors into focal adhesions (LaFlamme et al., 1992).

Several integrins bind their ligands solely when they are activated in response to physiological or nonphysiological stimulations. This process was called "inside-out" integrin signaling (Hynes, 1992). This "inside-out" signaling pathway modulates the affinities of the integrins for their ligands. This activation results from a conformational change of the receptors that is not fully understood. It has been well described for the β_2 integrins on neutrophils, monocytes, and lymphocytes, the β_1 integrins on lymphocytes, and the $\alpha_{IIb}\beta_3$ integrins on platelets. The increase in the affinity can be triggered by cell activators such as ADP, thrombin, inflammatory mediators, or phorbol esters, or it can also be induced by some monoclonal antibodies (O'Toole et al., 1990; Gulino et al., 1990; Kouns et al., 1990; Frelinger et al., 1991) and by the ligands themselves (Du et al., 1991). Whereas the cytoplasmic domain of α_{IIb} controls the affinity state of the extracellular domain of $\alpha_{IIb}\beta_3$ (O'Toole et al., 1991), the binding activity of $\alpha_L\beta_2$ is modulated by the cytoplasmic domain of the β_2 subunit (Hibbs et al., 1991a). Phosphorylation (of the integrin or of associated proteins) or lipid mediators (Hermanowski-Vosatka et al., 1992) may be involved in this regulation. However, only a small fraction of the $\alpha_{IIb}\beta_3$ receptors is phosphorylated, and this post-translational event remains of uncertain significance (Hillery et al., 1991; Shattil & Brugge, 1991). On the other hand, mutagenesis of the β_2 cytoplasmic domain can dissociate phosphorylation from activation in lymphoid cells (Hibbs et al., 1991b) and shed the suspicion about the involvement of a direct phosphorylation of integrins in inside-out signaling.

Integrins that are normally found with a constitutive high affinity toward their ligands can also be in equilibrium between a high and a low affinity conformational state. For instance, the activation of T-cells leads to the stimulation of the binding activities of β_1 integrins such as $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$. Consequently, an increase in cell adhesion onto collagen, fibronectin, and laminin is observed (Shimizu et al., 1990; Chan et al., 1991; Wilkins et al., 1991). Recent data have shown that the affinity of $\alpha_{6A}\beta_1$ and $\alpha_{6B}\beta_1$, two integrins with α_6 variants, can be regulated by "inside-out" signaling pathways after PMA stimulation of macrophages (Shaw et al., 1993). The modulation of β_1 integrins affinities is also described in many other cells: the activity of $\alpha_5\beta_1$ in keratinocytes (Adams & Watt, 1990) and $\alpha_6\beta_1$ in retinal neurons (Neugebauer & Reichardt, 1991) is inhibited during cell differentiation, without change in their expression levels.

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Although the molecular mechanisms of the control of inside-out signaling remain to be unraveled, it is noteworthy that the mobilization of intracellular calcium in T-cells, by ionophores or endoplasmic reticulum Ca^{2+}-ATPase inhibitors, plays an important role in the activation of T-cell β_1 integrins (Hartfield et al., 1993). Furthermore, the binding activities of $\alpha_5\beta_1$ and $\alpha_V\beta_3$ *in vitro* are regulated by cytosolic proteins and micromolar concentrations of intracellular calcium (Marie et al., 1991) whereas the activity of β_1 integrin *in vivo* is modulated by physiological levels of intracellular calcium in myelomonocytic U-937 cells (Arroyo et al., 1992). This suggests that some intracellular proteins and calcium might be involved in the inside-out transduction pathway. In order to identify those putative regulators, cytosolic proteins that bind synthetic peptides corresponding to the integrin cytoplasmic sequences were analyzed. One of them is calreticulin, an intracellular calcium-binding protein which can interact with the α subunits of integrins via the highly conserved KXGFFKR amino acid sequence present in the cytoplasmic domain of all integrin α subunits (Rojiani et al., 1991). Recent studies have shown that calreticulin copurifies with $\alpha_3\beta_1$, $\alpha_2\beta_1$, and $\alpha_5\beta_1$ integrins, after immunoprecipitation, and that antisense-calreticulin oligonucleotides inhibit the attachment and spreading of the cells plated on extracellular matrix substrates (Leung-Hagesteijn et al., 1994). Another protein, named integrin associated protein (IAP), is found physically and functionally associated with integrins (Brown et al., 1990). IAP is involved in the regulation of ligand binding by β3 integrins (Brown et al., 1990). This transmembrane protein has a single extracellular immunoglobulin domain, five transmembrane rods, and a short cytoplasmic tail (Lindberg et al., 1993). It is specifically required for the integrin-regulated calcium influx (Schwartz et al., 1993). The structural and functional data suggest that IAP might be an integrin-associated calcium channel.

In agreement with the idea that intracellular calcium may play a key role in integrin-mediated cell adhesion, it has been shown that repeated transient increases in intracellular calcium occur in motile neutrophils and act as signals that regulate the cycles of detachment needed for migration on adhesive surfaces (Marks & Maxfield, 1990). The inhibition of intracellular calcium transients impairs neutrophil motility on fibronectin and vitronectin (Marks et al., 1991). A specific peptide inhibitor of the calcium/calmodulin-dependent phosphatase calcineurin (protein phosphatase 2B, PP2B),[1] introduced into neutrophils, inhibits their migration onto vitronectin (but not onto fibronectin) by interfering with the release of the cells from sites of attachment. This inhibition can be reversed by blocking RGD-mediated attachment to the substrate (Hendey et al., 1992). These data suggest that activation of calcineurin by calcium regulates integrin-mediated motility onto vitronectin, whereas the motility on fibronectin requires other calcium-dependent effectors. It seems likely that this phosphatase activity regulates cell detachment either directly by dephosphorylating integrins or receptor-associated proteins or indirectly by initiating a cascade of other phosphoserine or phosphotyrosine phosphatases and kinases (Hendey & Maxfield, 1993).

In this study, an *in vitro* assay using Chinese hamster ovary cell lysate has been developed to study directly the interaction of $\alpha_5\beta_1$ with its substrate fibronectin, in a cytosolic environment. General phosphatase inhibitors used during cell lysis completely abolished the fibronectin/integrin interaction in our assay, indicating that the affinity of the fibronectin receptor might be modulated by a protein phosphatase activity. The requirement of intracellular calcium and the inhibition of the $\alpha_5\beta_1$ integrin/fibronectin interaction by specific phosphatase inhibitors and monoclonal antibodies raised against calcineurin suggested that the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin allowed the interaction between the fibronectin receptor and its substrate. Further investigations indicated that $\alpha_5\beta_1$ itself is not the direct substrate of calcineurin and suggested that one substrate of calcineurin may regulate the integrin affinity by interacting directly or indirectly with the fibronectin receptor.

EXPERIMENTAL PROCEDURES

Cell Culture. Chinese hamster ovary cells clone 15B (CHO 15B) were grown on plates in the minimum essential medium with α modification (α MEM) without nucleoside and supplemented with 7.5% fetal calf serum (v/v) at 37 °C, in a humidified 5% CO_2/95% air atmosphere. Cells were harvested with phosphate buffer saline (PBS) supplemented with 1 mM EDTA and 0.05% trypsin (w/v).

Antibodies. In the solid phase assay integrins were detected either with a rabbit polyclonal antiserum directed against the C-terminus of the α_5 subunit (Chemicon International Inc) or with a rabbit polyclonal antiserum directed against the whole $\alpha_5\beta_1$ receptor (GIBCO BRL). The monoclonal antibody raised against calcineurin β subunit was supplied from UBI. PB1, a monoclonal antibody raised against hamster $\alpha_5\beta_1$ integrin, is a generous gift of Dr. Juliano (Chapel Hill University, NC). Control mouse IgG1κ antibodies were supplied from Sigma. Immunoprecipitations of the fibronectin receptor were carried out with the affinity-purified rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum from GIBCO BRL.

Cell Lysate. CH0 15B cells were harvested from 100 mm tissue culture dishes with trypsin/EDTA and washed once with α MEM supplemented with 7.5% fetal calf serum (v/v). Cells were then washed twice with PBS at 4 °C and lysed for 1 h at 4 °C in PBS containing 1% Triton X100, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 10 μg/mL leupeptine, and 10 μM pepstatin A. In some experiments phosphatase inhibitors or calmodulin inhibitors were added into the lysis buffer just before the lysis of the cells. The cell lysate was clarified by centrifugation at 10000*g* for 20 min at 4 °C. Its protein concentration was determined by the micro BCA protein assay (Pierce).

Solid Phase Assay. Microtiter plates (96-well; MaxiSorp Immuno Plate, Nunc) were coated overnight at room temperature with 200 μL of fibronectin at 25 μg/mL. Fibronectin was purified from bovine plasma according to the method of Engvall and Ruoslahti (1977). The wells were washed twice with PBS and blocked with 300 μL of PBS

[1] Abbreviations: α MEM, minimum essential medium with α modification; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); BSA, bovine serum albumin; CHO 15B, Chinese hamster ovary cells clone 15B; DMEM, Dulbecco's modified Eagle's medium; IAP, integrin associated protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PMSF, phenylmethanesulfonyl fluoride; PP1, PP2A, PP2B, and PP2C, protein phosphatases 1, 2A, 2B, and 2C; SDS, sodium dodecyl sulfate.

and 1% BSA (w/v), 1 h at room temperature. Appropriate dilutions of cell lysate under 200 μL were incubated in fibronectin-coated wells for 60 min at 37 °C. Nonbound proteins were removed by four washes with PBS, 0.1% Tween 20, and 1% BSA. $\alpha_5\beta_1$ integrin or integrin α_5 subunit was detected with 100 μL of a primary polyclonal antibody, 1 h at 37 °C. The rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum was used at the dilution of 1/400 and the rabbit anti-integrin α_5 subunit antiserum at the dilution of 1/5000. After four washes with PBS, 0.1% Tween 20, and 1% BSA, 100 μL of anti-rabbit IgG–biotin conjugate F(ab')$_2$ fragment (Boehringer, Manheim) at a dilution of 1/2000 was added during 30 min at 37 °C. After four washes with PBS, 0.1% Tween 20, and 1% BSA, 100 μL of streptavidin–horseradish peroxidase conjugate (Boehringer, Manheim) at a dilution of 1/10 000 was added for 30 min at 37 °C. After four washes with PBS, 100 μL of peroxidase substrate (TMB Peroxidase EIA Substrate Kit from Bio-Rad Laboratories) was added. After 4 min at room temperature, reaction was stopped by adding 100 μL per well of 1 N H_2SO_4 and enzyme activity was measured at 450 nm with a photometer (Dynatech MR5000). Specific binding of the fibronectin receptor to the coated fibronectin was calculated by subtracting the nonspecific signal obtained when lysis buffer was incubated onto fibronectin-coated well from the signal obtained when cell lysate is incubated under the same conditions.

Phosphatase Activity. Total phosphatase activity was measured in cell lysate pretreated with different phosphatase inhibitors. A volume of 100 μL of clarified cell lysate corresponding to 200 μg of proteins was incubated with 2 mM *p*-nitrophenyl phosphate for 40 min at 37 °C. Phosphatase activity was monitored by following the absorbance at 405 nm, due to the release of *p*-nitrophenol.

Radioactive Labeling of the Cells. CHO 15B cells were radiolabeled either with [^{35}S]methionine–cysteine (L-[^{35}S] *in vitro* cell labeling mix from Amersham) or with [^{32}P]-orthophosphate (ICN Biomedicals). Metabolic labeling was carried out with adherent cells that were washed twice with PBS at 37 °C and incubated in DMEM without methionine and cysteine (Flow Laboratories) for 30 min at 37 °C. Then, the [^{35}S]methionine–cysteine mix was added (200 μCi/100 mm tissue culture dish), and the incubation was continued for 6 h. After one wash with DMEM without methionine and cysteine, the cells were harvested, washed, and lysed as described above. Labeling with [^{32}P] was performed on adherent cells. The cells were washed twice with DMEM without sodium phosphate (Sigma) at 37 °C and were incubated in the same medium for 30 min at 37 °C. Then, [^{32}P]orthophosphate was added (1 mCi/100 mm tissue culture dish), and the cells were further incubated for 3 h at 37 °C. After three more washes with DMEM without sodium phosphate, the cells were harvested, washed, and lysed as described above.

Immunoprecipitation. A clarified lysate of the labeled cells was incubated at 37 °C during 30 min. Then, is was incubated with specific integrin antibodies for 2 h at 4 °C under gentle agitation. Immune complexes were bound to protein G–agarose (Calbiochem) for 1 h at 4 °C with 50% (v/v) protein G–agarose beads in suspension in the lysis buffer. Before use, the beads were blocked with a cell lysate from CHO 15B cells. At the end of the incubation time, the beads were washed three times with a high salt buffer (500 mM NaCl, 0.1% SDS, 0.5% Triton X100, 50 mM Tris, pH 8) to remove the unbound proteins and twice with a low salt buffer (0.1% SDS, 0.5% Triton X100, 50 mM Tris, pH 8) to improve the subsequent gel electrophoresis. The labeled immunoprecipitated proteins were resolved by SDS–PAGE under nonreducing conditions. To shorten exposure times of X-ray films, the gels containing [^{35}S]-labeled proteins were treated with the autoradiography enhancer EN^3-HANCE (NEN Research Products, DuPont). Gels were dried and fluorography was carried out at −80 °C using autoradiography films (Hyperfilm-MP from Amersham).

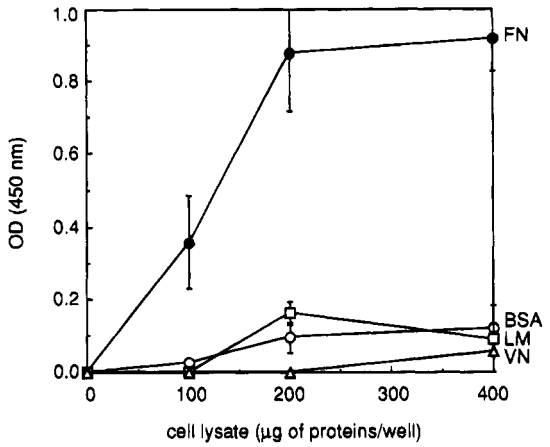

FIGURE 1: Binding of the fibronectin receptor to fibronectin-coated plastic in the presence of Ca^{2+} and Mg^{2+}. Microtiter wells were coated with 25 μg/mL fibronectin (FN), 10 μg/mL laminin (LM), 10 μg/mL vitronectin (VN), or 1 mg/mL bovine serum albumin (BSA) and incubated with increasing concentrations of cell lysate in the presence of Ca^{2+} and Mg^{2+} for 60 min at 37 °C. Bound fibronectin receptors were detected by a rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum as described under Experimental Procedures. Mean and standard error from two experiments are shown.

Immunoblot. Proteins from CHO 15B cell lysate were separated on a 7.5% polyacrylamide gel under reducing conditions. The proteins were then transferred electrophoretically to a nitrocellulose filter and detected with a monoclonal antibody raised against the calcineurin β subunit. Staining was performed using a peroxidase-labeled second antibody and the ECL chemiluminescent kit from Amersham.

RESULTS

The $\alpha_5\beta_1$ Integrin Binds Specifically Fibronectin in a Solid Phase Assay. When increasing protein concentrations of a cell lysate were incubated in the presence of calcium and magnesium into fibronectin coated 96-multiwell plates, a dose-dependent binding of the fibronectin receptor ($\alpha_5\beta_1$) onto the coated plastic was immunodetected by specific antibodies. The binding of $\alpha_5\beta_1$ reached a plateau when 200 μg of protein was incubated into the fibronectin coated wells (Figure 1).

The optimal coating concentration of fibronectin was 25 μg/mL (data not shown). Therefore, this coating concentration together with an amount of proteins of 300 μg/well was used in all the subsequent experiments. The assay was quite specific since only a minimal binding of fibronectin receptor was detected when fibronectin was replaced by laminin or vitronectin. This binding corresponded to the nonspecific binding of the integrin onto bovine serum albumin (Figure 1). Moreover, the omission of either the primary antibody, the second antibody coupled to biotin, or both eliminated the signal (data not shown).

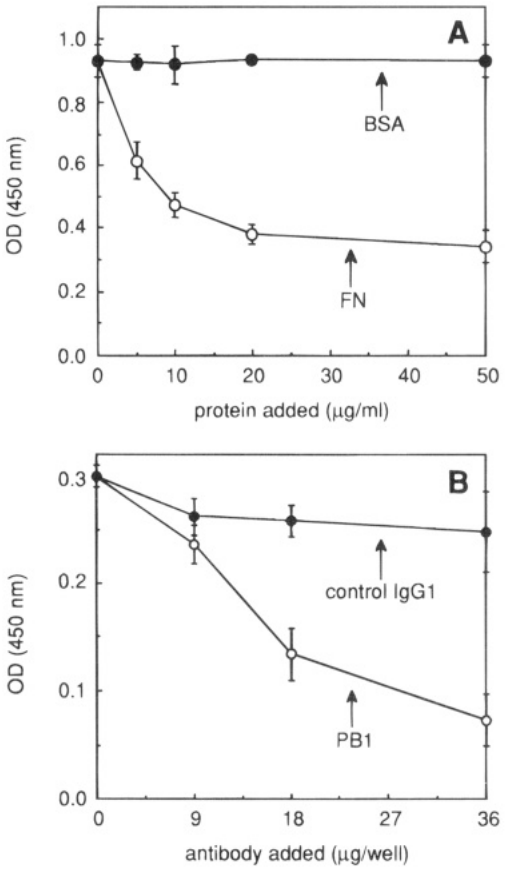

FIGURE 2: Specific inhibition of the fibronectin receptor binding to fibronectin-coated plastic in the presence of Ca^{2+} and Mg^{2+}. A constant amount of cell lysate (300 μg of proteins) was preincubated with various concentrations of (A) soluble fibronectin (FN) or bovine serum albumin (BSA) or (B) mouse anti-$\alpha_5\beta_1$ monoclonal antibody (PB1) or control mouse IgG1κ antibody (control IgG1), for 30 min at 4 °C before the incubation in fibronectin-coated wells in the presence of Ca^{2+} and Mg^{2+}. Bound fibronectin receptors were detected either by a rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum (A) or by a rabbit anti-C-terminus α_5 subunit polyclonal antiserum (B). Mean and standard error from two experiments are shown.

Fibronectin Receptor Binding to Coated Fibronectin Is Inhibited by Soluble Fibronectin and Anti-Fibronectin Receptor Antibodies. To further control the specificity of our assay, soluble fibronectin and PB1 (a monoclonal antibody raised against the fibronectin receptor) were tested for their capability to inhibit the binding of $\alpha_5\beta_1$ to the fibronectin-coated plastic. Both soluble fibronectin and PB1 competed with the fibronectin receptor for binding. Half-maximal inhibition was obtained with approximately 5 μg/mL soluble fibronectin (Figure 2A) or 14 μg/well mouse IgG1 antibody PB1 (Figure 2B). Under identical experimental conditions, no inhibition of the fibronectin receptor binding to coated fibronectin was detected in the presence of 50 μg/mL BSA (Figure 2A) and only a small inhibition was observed in the presence of 36 μg/well control mouse IgG1 (Figure 2B).

The Interaction between the Fibronectin Receptor and Its Ligand Requires Intracellular Calcium in Vitro. In vivo, the binding of integrins onto their ligands requires divalent cations such as calcium or magnesium. Cation binding sites are localized on the extracellular domain of the integrin α subunit, in a region contributing to the ligand-binding domain. The binding of extracellular cations is essential for the receptor function, and the nature of the divalent cation can influence both the affinity and the specificity of the

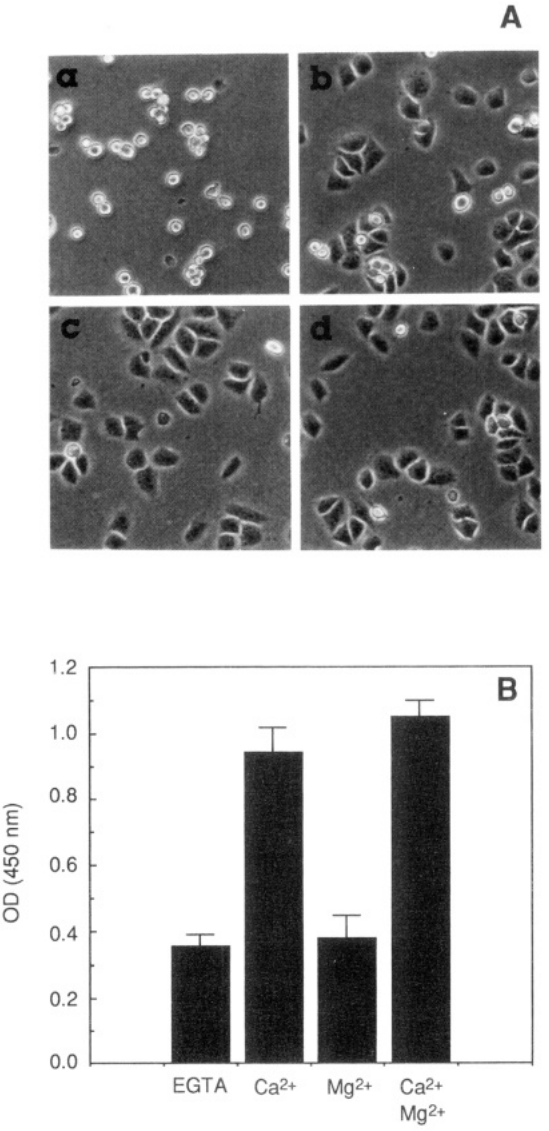

FIGURE 3: Effect of Ca^{2+} and Mg^{2+} on the binding activity of the fibronectin receptor *in vivo* and *in vitro*. (A) Phase contrast micrographs of CHO 15B cells after 1 h of incubation at 37 °C onto fibronectin-coated Petri dishes in phosphate buffer saline in the absence of divalent cations (a) and in the presence of 1 mM $CaCl_2$ (b), 1 mM $MgCl_2$ (c), or 1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (d). Original magnification 40×; the original photograph as been reduced to 65% of its original size for publication purposes. (B) A constant amount of cell lysate (300 μg of proteins per well) pretreated with 1 mM EGTA (EGTA), 1 mM $CaCl_2$ (Ca^{2+}), 1 mM $MgCl_2$ (Mg^{2+}), and 1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (Ca^{2+}-Mg^{2+}) was incubated in fibronectin-coated wells. Bound fibronectin receptors were detected by a rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum. Mean and standard error from two experiments are shown.

integrin (Gailit & Ruoslahti, 1988; Kirchhofer et al., 1990, 1991). *In vitro*, it was shown that the binding of liposomes containing purified fibronectin receptors onto coated fibronectin was identical in the presence of calcium, magnesium, or both, at a final concentration of 1 mM each (Gailit & Ruoslahti, 1988). Herein, we compared the effect of calcium and magnesium on the binding *in vivo* and *in vitro* of the fibronectin receptor onto fibronectin-coated plastic. As shown in Figure 3A, CHO 15B cells attached and spread onto coated fibronectin when calcium, magnesium, or both were present at the concentration of 1 mM, but failed to bind onto the fibronectin-coated plastic in the absence of divalent cations. On the other hand, Figure 3B showed that in our

solid phase assay with CHO 15B cell lysate, no interaction between the fibronectin receptor and the coated fibronectin was detected when either 1 mM magnesium alone or EGTA was added. The latter result pointed out a major difference between purified fibronectin receptors that bind fibronectin with the magnesium alone (Gailit & Ruoslahti, 1988) and the fibronectin receptor surrounded by other cellular proteins in our assay. Under these conditions, $\alpha_5\beta_1$ in CHO 15B cell lysate failed to bind fibronectin with magnesium alone and required calcium, although the whole cells did not need calcium to bind and spread onto coated fibronectin. These experiments strongly suggested that intracellular calcium in association with intracellular calcium-binding protein(s) such as calmodulin might modulate the fibronectin receptor affinity for its ligand *in vitro*. Most often, calcium regulation involved the action of calmodulin-stimulated protein kinases and/or phosphatases (Cohen, 1982; Nairn et al., 1985). This hypothesis was tested by analyzing the effects of general phosphatase inhibitors in our assay.

The Interaction between $\alpha_5\beta_1$ and Fibronectin in Vitro Is Controlled by a Protein Phosphatase. Calmodulin controls both protein kinases and calcium-dependent phosphatases. The potential role of calcium/calmodulin protein kinases was investigated using KN-62, a specific inhibitor for this class of enzymes. At the very high concentration of 20 μM, no significant effect was observed in our solid phase assay (data not shown). Consequently, the involvement of protein phosphatases in the regulation of $\alpha_5\beta_1$ integrin affinity in the assay was tested by adding general phosphatase inhibitors such as disodium phosphate or β-glycerophosphate directly into the lysis buffer. Such inhibitors are currently used in experiments to maintain the proteins in their phosphorylated state (Cicirelli et al., 1988; Martin-Pérez et al., 1986). One of them, disodium phosphate, inhibited the interaction between the fibronectin receptor and its ligand (Figure 4A) with a half-maximum inhibition reached at the final concentration of 17 mM. A similar inhibition was obtained with β-glycerophosphate (data not shown). In Figure 4B is shown that, indeed, the phosphatase activity in the cell lysate was actually reduced when disodium phosphate was added. The addition of disodium phosphate into the lysis buffer corresponded to a decrease in the total phosphatase activity which can be perfectly correlated with the inhibition of the integrin/fibronectin interaction monitored in Figure 4A. No inhibitory effect of disodium phosphate or β-glycerophosphate on the $\alpha_5\beta_1$/fibronectin interaction was detected when these two general phosphatase inhibitors were added in the cell lysate 30 min after the beginning of the incubation (data not shown). This control indicated that the inhibition of disodium phosphate and β-glycerophosphate in our assay was specific and was not the result of Ca^{2+} chelation. The involvement of protein phosphatases in the regulation of fibronectin/integrin interaction was also tested by adding ATPγS, a nonhydrolyzable analogue of ATP, to the lysis buffer. At the concentration of 1 mM, ATPγS inhibited the binding of the fibronectin receptor onto the fibronectin-coated plastic in our assay, whereas 1 mM ATP had no effect (Figure 5). Altogether, our results strongly suggested that the interaction between the fibronectin receptor and its ligand was controlled in CHO cell lysates by a phosphatase activity. The decrease in this activity reduced the affinity of $\alpha_5\beta_1$ for fibronectin under our experimental conditions.

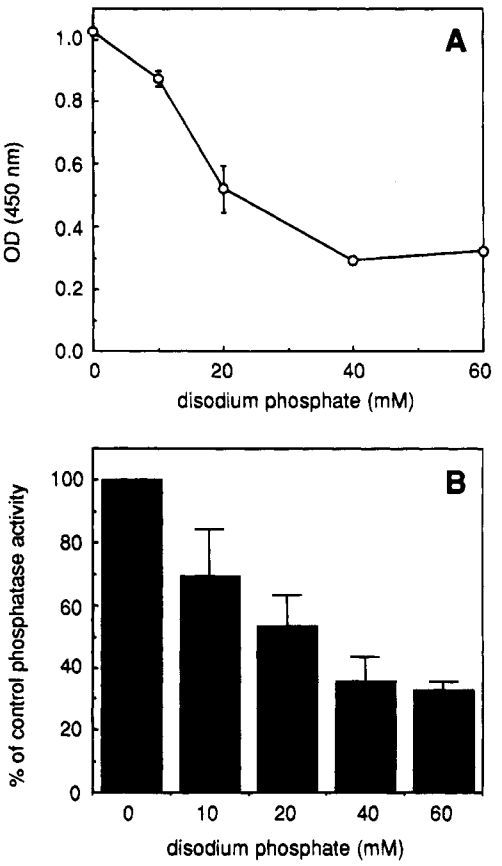

FIGURE 4: Effect of disodium phosphate on the binding of the fibronectin receptor to fibronectin-coated plastic. (A) A constant amount of cell lysate (300 μg of proteins) pretreated with various concentrations of disodium phosphate was incubated in fibronectin-coated wells. Bound fibronectin receptors were detected by a rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum. Mean and standard error from two experiments are shown. (B) Total phosphatase activity was measured in a constant amount of cell lysate (200 μg of proteins) pretreated with various concentrations of disodium phosphate as described under Experimental Procedures. The results are expressed as a percentage of control phosphatase activity in the absence of disodium phosphate. Mean and standard error from two experiments are shown.

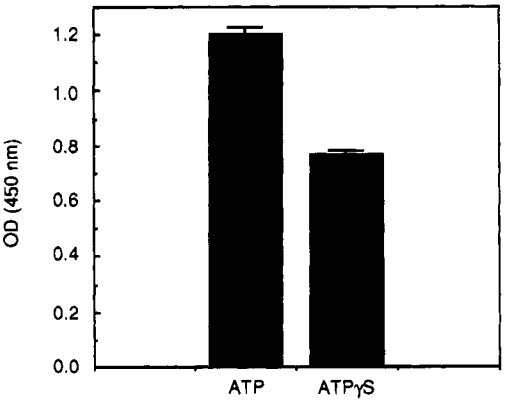

FIGURE 5: Effect of ATPγS on the binding of the fibronectin receptor to fibronectin-coated plastic. A constant amount of cell lysate (300 μg of proteins) pretreated with 1 mM ATP (ATP) and 1 mM ATPγS (ATPγS) was incubated in fibronectin-coated wells. Bound fibronectin receptors were detected by a rabbit anti-$\alpha_5\beta_1$ integrin antiserum. Mean and standard error from two experiments are shown.

In Vitro the $\alpha_5\beta_1$/Fibronectin Interaction Is Regulated by a Calcium/Calmodulin-Dependent Protein Phosphatase. Distinct major families of phosphatases have been described

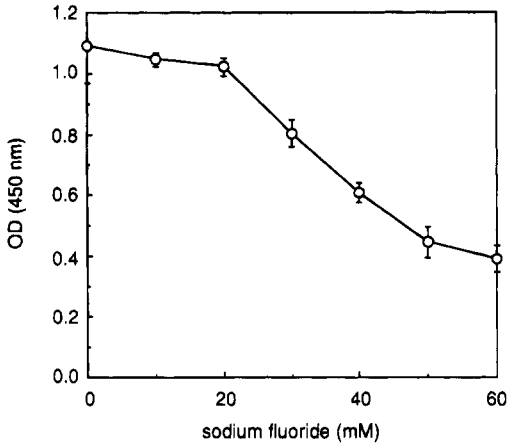

FIGURE 6: Effect of sodium fluoride on the binding of the fibronectin receptor to fibronectin-coated plastic. A constant amount of cell lysate (300 μg of proteins) pretreated with various concentrations of sodium fluoride was incubated in fibronectin-coated wells. Bound fibronectin receptors were detected by a rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum. Mean and standard error from three experiments are shown.

to date: tyrosine phosphatases and four well-known types of serine/threonine phosphatases. Tentative identification of the type of phosphatase involved in the regulation of integrin/fibronectin interaction was carried out with more specific phosphatase inhibitors introduced into the lysis buffer. Sodium fluoride was used first. Unlike many serine/threonine protein phosphatases which are inhibited by NaF, protein tyrosine phosphatases appeared to be resistant to fluorides. Furthermore, PP2C, the magnesium-dependent serine/threonine protein phosphatase, is also characterized by its relative resistance to NaF (Ingebritsen et al., 1983). Figure 6 showed the NaF was a potent inhibitor of the integrin/fibronectin interaction with a half-maximum inhibition at the concentration of 35 mM. This result indicated that the phosphatase involved in the regulation of $\alpha_5\beta_1$/fibronectin interaction in our assay was neither a tyrosine phosphatase nor PP2C.

However, one should keep in mind that although NaF is a potent inhibitor of some protein phosphatases, the presence of F^- may impair the function of trimeric G proteins. The complex of F^- with Al^{3+}, a metal ion that is often supplied in sufficient quantities in standard laboratory water and from glassware, forms an active ion, AlF_4^-, which binds the GDP-bound form of some GTP-binding proteins and mimics the γ-phosphate of GTP (Chabre, 1990). In order to rule out the hypothesis that NaF could inhibit the integrin/fibronectin interaction by activating a heterotrimeric G protein, GTPγS, a nonhydrolyzable analogue of GTP, was added to the lysis buffer. Even at the final concentration of 100 μM GTPγS, which is a concentration known to activate G proteins *in vitro* (Melançon et al., 1987), the $\alpha_5\beta_1$ integrin bound the fibronectin-coated plastic (data not shown). Thus, activation of a G protein did not appear to play any significant role in the regulation of the integrin/fibronectin interaction in our assay.

To further specify the type of phosphatase involved in the regulation of $\alpha_5\beta_1$ affinity, two other phosphatases inhibitors were tesed. Okadaic acid, a specific inhibitor of serine/threonine phosphatases PP1 and PP2A, and sodium vanadate, an inhibitor of serine/threonine phosphatases PP1, PP2A, and PP2C and of most tyrosine phosphatases (Shenolikar & Nairn, 1991), were added into the lysis buffer. As shown in Figure 7A, no significant inhibition of the integrin/fibronectin interaction was detected in the presence of each of these two phosphatase inhibitors. However, okadaic acid and sodium vanadate inhibited the total phosphatase activity of the cell lysates by 43% and 71%, respectively (Figure 7B). These different extents of inhibition corresponded to a broader spectrum of inhibition among phosphatases of sodium vanadate as compared to okadaic acid. On the other hand, 60 mM NaF, which fully inhibited the integrin/fibronectin interaction in our solid phase assay (Figure 7A), reduced the total phosphatases activities in our cell lysates by only 18% (Figure 7B). This suggests that the phosphatase activity inhibited by NaF and involved in our assay is only a minor fraction of the total phosphatases activities present in the cell lysate.

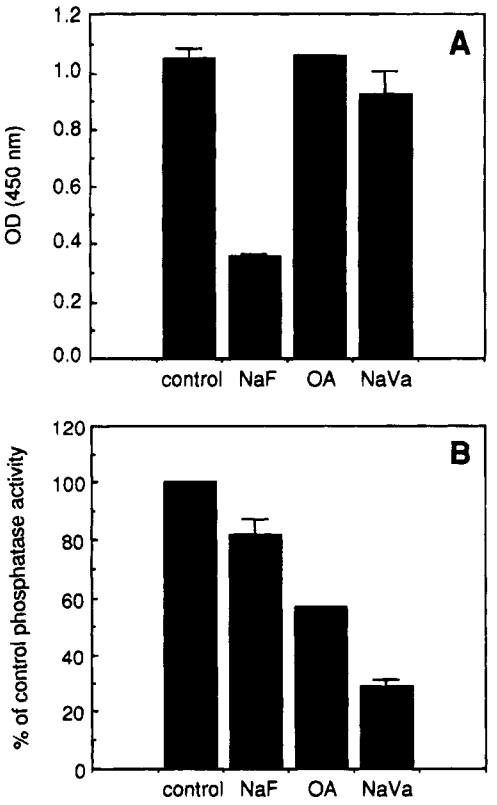

FIGURE 7: Effect of different phosphatase inhibitors on the binding of the fibronectin receptor to fibronectin-coated plastic. (A) A constant amount of cell lysate (300 μg of proteins) nontreated (control) or pretreated with 60 mM sodium fluoride (NaF), 10 μM okadaic acid (OA), and 1 mM sodium vanadate (NaVa) was incubated in fibronectin-coated wells. Bound fibronectin receptors were detected by a rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum. Mean and standard error from two experiments are shown. (B) Total phosphatase activity was measured in a constant amount of cell lysate (200 μg of proteins) nontreated (control) or pretreated with 60 mM sodium fluoride (NaF), 10 μM okadaic acid (OA), and 1 mM sodium vanadate (NaVa). The results are expressed as a percentage of control phosphatase activity in the absence of phosphatase inhibitor. Mean and standard error from three experiments are shown.

The inhibition pattern found with the different phosphatase inhibitors used and the involvement of calmodulin and intracellular calcium strongly suggested that the serine/threonine calcium/calmodulin-dependent protein phosphatase calcineurin (PP2B) regulated fibronectin binding to $\alpha_5\beta_1$ *in vitro*. Finally, using an anti-calcineurin monoclonal antibody, we were able to verify the specificity of this antibody,

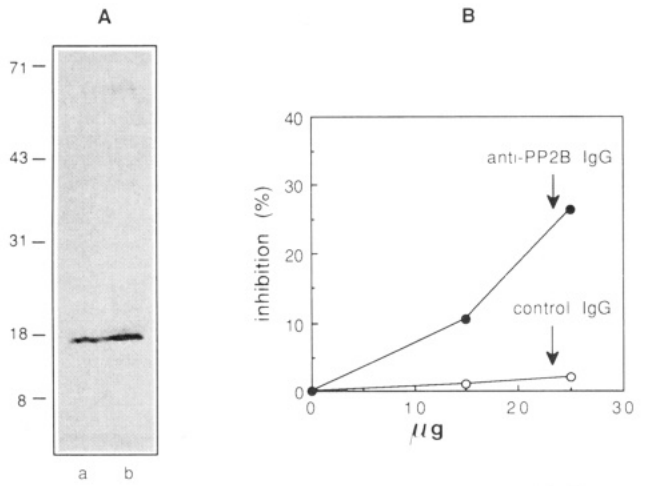

FIGURE 8: Inhibition of the fibronectin receptor binding to fibronectin-coated plastic by a specific monoclonal antibody raised against calcineurin. (A) Immunoblot of CHO 15B cell lysate with anti-calcineurin β subunit monoclonal antibody. 128 μg (a) or 256 μg (b) of proteins from a crude cell lysate were run on a 7.5% polyacrylamide gel under reducing conditions. Positions of molecular weight markers (in kDa) are indicated. (B) A constant amount of cell lysate (300 μg of proteins) was preincubated with various concentrations of mouse anti-calcineurin β subunit monoclonal antibody (anti-PP2B IgG) or control mouse IgG antibody (control IgG), for 30 min at 4 °C before the incubation in fibronectin-coated wells. Bound fibronectin receptors were detected by a rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum.

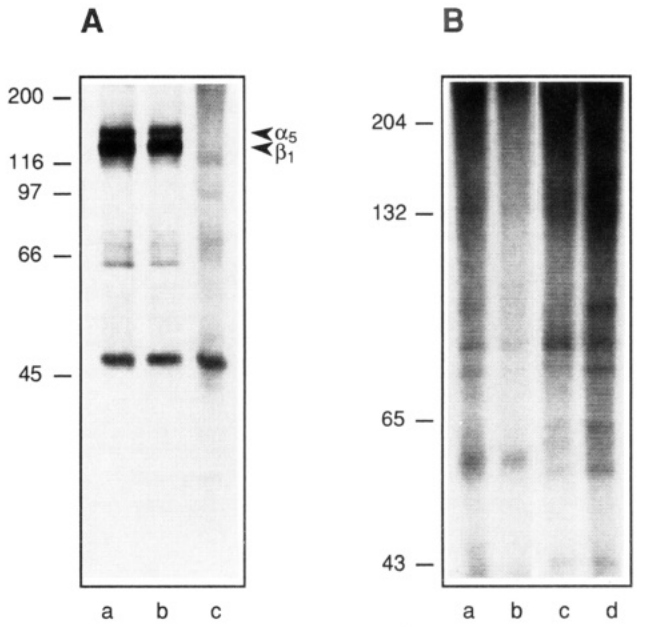

FIGURE 9: Immunoprecipitation of the $\alpha_5\beta_1$ integrin after metabolic labeling of Chinese hamster ovary cells. (A) Lysate from [^{35}S]-methionine−cysteine-labeled cells were used for immunoprecipitation as described under Experimental Procedures. Immunoprecipitations from control (lanes a and c) or NaF-treated cell lysates (lane b) were carried out using a rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum (lanes a and b) or a preimmune serum (lane c). (B) Lysate from [^{32}P]-labeled cells was used for immunoprecipitation as described under Experimental Procedures. Immunoprecipitations from control (lanes a and b) or NaF-treated cell lysates (lanes c and d) were carried out using a rabbit anti-$\alpha_5\beta_1$ integrin polyclonal antiserum (lanes a and c) or a preimmune serum (lanes b and d). Samples were analyzed on a 10% polyacrylamide gel (A) and a 7.5% polyacrylamide gel (B) under nonreducing conditions. Positions of the $\alpha_5\beta_1$ integrin and molecular weight markers (in kDa) are indicated.

together with the presence of calcineurin in our cell extracts (Figure 8A). Moreover, when preincubated with the cell lysates, the anti-calcineurin antibody was able to induce a moderate but dose-dependent inhibition of the $\alpha_5\beta_1$ binding onto fibronectin in our assay (Figure 8B). The effects of the calcium/calmodulin-dependent phosphatase calcineurin could be caused indirectly by a yet unidentified molecule, or by a chemical modification of the integrin cytoplasmic domains themselves. The presence of conserved tyrosine, threonine, and serine residues in the cytoplasmic domains of integrin subunits suggests that phosphorylation might regulate the affinity state of the integrin.

The $\alpha_5\beta_1$ Integrin Is Not the Substrate of the Calcium/Calmodulin-Dependent Protein Phosphatase. In order to study the possible interaction of the integrin with a protein regulator dephosphorylated by calcineurin, the immunoprecipitation of the $\alpha_5\beta_1$ integrin was performed after the metabolic labeling of CHO 15B cells. Figure 9A showed that in [^{35}S]methionine−cysteine-labeled cells, the fibronectin receptor could be immunoprecipitated by an immunopurified rabbit anti-$\alpha_5\beta_1$ polyclonal antibody from NaF-treated or control cell lysates. Although small minor components coimmunoprecipitated with the fibronectin receptor, no significant differences were detected in the presence or in the absence of the phosphatase inhibitor NaF.

Using the same antibody, the immunoprecipitation was also carried out with [^{32}P]-labeled cells. With or without NaF, the phosphorylation of either α and β subunits was never detected (Figure 9B) although a different pattern of phosphorylation was obtained under the two experimental conditions. Our experiments clearly indicated that $\alpha_5\beta_1$ remained mostly unphosphorylated and was not the target of phosphorylation/dephosphorylation cascades involving calcineurin and leading to the modulation of the affinity of this integrin.

DISCUSSION

Herein, we describe an *in vitro* assay that allows us to estimate the interaction between the fibronectin receptor $\alpha_5\beta_1$ and its ligand fibronectin. Earlier studies have already shown that integrin/ligand interaction can be carried out in a solid phase assay. The originality of our assay is that the integrin/ligand interaction can be performed directly from a crude cell lysate without any purification step of the integrin receptor. Moreover, it allows us to measure the binding activity of the receptor on its ligand in the environment of cytosolic proteins. Although the starting biological material was a crude cell extract, our assay was quite specific since only fibronectin allowed the detection into the coated wells of either $\alpha_5\beta_1$ (Figure 1) or α_5 chain (Figure 2B) by specific antibodies. Additionally, the monoclonal inhibitory antibody PB1 raised against the hamster fibronectin receptor (Brown & Juliano, 1985) blocked the assay (Figure 2B). However, it is likely that the use of CHO cells improved the quality of the assay since these cells displayed a limited number of integrins, $\alpha_5\beta_1$ being the major one expressed at the cell surface (personal results). Finally, the interaction between fibronectin-coated plastic and $\alpha_5\beta_1$ was reversible since soluble fibronectin competed with $\alpha_5\beta_1$ for binding (Figure 2A).

Surprisingly, $\alpha_5\beta_1$ in our assay displayed distinct features as compared to cell/fibronectin interactions *in vivo*, or to the binding of the purified receptor on its ligand *in vitro* (Gailit & Ruoslahti, 1988). While magnesium or calcium could

equally well sustain the binding activity of the cell surface expressed fibronectin receptor or of the purified receptor, calcium was required in our assay (Figure 3). This major difference was not observed in a solid phase assay involving the purified fibronectin receptor (Gailit & Ruoslahti, 1988). As integrins bind their ligand in a divalent cation dependent manner and can accommodate either magnesium or calcium ions on their external cation binding sites, it was important to determine whether the calcium dependence of the *in vitro* assay was a trivial result of the magnesium/calcium exchange on the extracellular binding site or a specific intracellular effect of this ion. Therefore, whole cell binding assays were performed to address this question. On whole cells, it was possible that low external calcium concentrations would decrease the intracellular calcium stores, but it had no effect on cell binding. Thus, external calcium could be replaced by magnesium without any modification of the binding properties of $\alpha_5\beta_1$. It is likely that the calcium dependence of our assay relies on an intracellular process.

This hypothesis is consistent with previous data indicating that, in streptolysin O-perforated cells, the affinity state of the fibronectin receptor is modulated by intracellular proteins and calcium (Marie et al., 1991). Indeed, ATPγS mimicked the action of general phosphatase inhibitors such as disodium phosphate or β-glycerophosphate (Figures 4 and 5). Additionally, the action of more specific phosphatase inhibitors (Figures 6 and 7) revealed that the phosphatase activity involved in this process belongs to the calcineurin type (PP2B), a phosphatase that is calcium and calmodulin dependent. Finally, a specific anti-calcineurin monoclonal antibody inhibited the assay (Figure 8). Thus, the blockade of calcineurin activity either by sodium fluoride or by other inhibitors would result in the switch of $\alpha_5\beta_1$ from a high affinity state to a low affinity state. However, one could be concerned about the possibility that phosphorylated compounds or even NaF might chelate calcium in the lysis buffer and, consequently, would result in a nonspecific inhibition unrelated to any phosphatase activity. This artifact can be ruled out since a preincubation at 37 °C for 30 min of the cell lysate before the addition of these molecules annihilated their inhibitory effect on the integrin/fibronectin interaction, but not their chelating power toward calcium ions. Moreover, in our *in vitro* model we were routinely using 1 mM of calcium. However, no modification of the assay was observed between 0.5 and 1 mM of calcium, indicating that this high concentration was saturating all the biological systems.

Addition of ATP in the assay at a concentration up to 1 mM had no significant effect in the assay. Thence, no ATP was added to the cell lysate. It is probable that phosphorylation was occurring during cell lysis using endogenous ATP. It is likely that ATP was quickly converted into ADP in the cell lysates, inhibiting further phosphorylation. This hypothesis is fairly consistent with the lack of inhibition of NaF when it was added afterward.

A simple working hypothesis is that, during cell lysis in Triton X100, an activation of specific kinases occurred, initiating some phosphorylation cascade of the inside-out signaling pathway. Within the cell lysate, these phosphorylations could be specifically counteracted by calcium and calmodulin activated calcineurin. Inhibition of this phosphatase would result in the imbalance of the phosphorylation levels and eventually in the conformational switch of $\alpha_5\beta_1$ to a low affinity state. This working model is consistent with the fact that phosphatase inhibitors were no longer able to induce any loss of the integrin affinity for its ligand after a preincubation of the cell lysate at 37 °C for 30 min. Indeed, protein kinases are often very unstable in Triton X100 containing buffers. The preincubation period is likely to be sufficient to inactivate the protein kinases. Therefore, the calcineurin activity would no longer be required to maintain a low level of phosphorylation linked to the high affinity state of $\alpha_5\beta_1$.

Since we have designed experimental conditions under which the $\alpha_5\beta_1$ integrin could be in either a high or a low affinity state, it was possible to give an answer as to whether the integrin itself was the target or not of the kinases and/or calcineurin. Immunoprecipitation of the fibronectin receptor from the cell lysate labeled with [^{32}P] and treated with sodium fluoride revealed that both α_5 and β_1 chains were not phosphorylated. Thus the integrin was obviously not the primary target of the phosphorylation cascade involved in inside-out signaling. The conformational change of the integrin should result from an indirect interaction with some phosphorylated/dephosphorylated molecule. Yet, no protein coimmunoprecipitated with the fibronectin receptor (Figure 9) in a stoichiometric manner. Many interpretations can be provided to explain this. One may assume that the conformational states of the integrins are not in equilibrium. The switch between one conformational state and another would be achieved by a transient interaction with an intracellular regulatory molecule. Alternatively, a regulatory molecule with a low affinity for the integrin might not be immunoprecipitated with the $\alpha_5\beta_1$. Finally, the putative regulatory molecule could be a lipid (Hermanowski-Vosatka et al., 1992) or another nonproteinaceous molecule that might not be well resolved in our SDS−PAGE.

Ultimately, one has to consider whether the indirect control of calcineurin on the $\alpha_5\beta_1$ integrin, that occurred *in vitro*, is relevant to any biochemical process that belongs to the inside-out pathway that occurred *in vivo*. The exciting findings showing that transient increases in intracellular calcium acted as signals that regulated the cycles of detachment needed for the migration of neutrophils on fibronectin and vitronectin (Marks & Maxfield, 1990; Marks et al., 1991) and that a specific peptide inhibitor of the calcium/calmodulin-dependent phosphatase calcineurin blocked the neutrophil migration onto vitronectin (Hendey et al., 1992) support the idea that the calcineurin-dependent regulation occurs under physiological conditions. However, in neutrophils, the inhibitory peptide of calcineurin had no effect on the migration onto fibronectin, whereas under our experimental conditions, calcineurin seems to control the fibronectin receptor $\alpha_5\beta_1$. It is plausible that neutrophils use a functional fibronectin receptor distinct from $\alpha_5\beta_1$, with a different inside-out signaling pathway. In any case, our *in vitro* assay is a powerful tool to unravel the biochemical mechanisms of integrin control and pinpoint the key role of calcineurin in this process.

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REFERENCES

Adams, J. C., & Watt, F. M. (1990) *Cell 63*, 425−435.

Arroyo, A. G., Sanchez-Mateos, P., Campanero, M. R., Martin-Padura, I., Dejana, E., & Sanchez-Madrid, F. (1992) *J. Cell Biol. 117*, 659−670.

Brown, E. J., & Juliano, R. L. (1985) *Science 228*, 1448−1451.

Brown, E. J., Hooper, R. L., Ho, T., & Gresham, H. (1990) *J. Cell Biol. 111*, 2785−2794.

Chabre, M. (1990) *Trends Biochem. Sci. 15*, 6−10.

Chan, B. M., Wong, J. G., Rao, A., & Hemler, M. E. (1991) *J. Immunol. 147*, 398−404.

Cicirelli, M. F., Pelech, S. L., & Krebs, E. G. (1988) *J. Biol. Chem. 263*, 2009−2019.

Cohen, P. (1982) *Nature 296*, 613−620.

Du, X. P., Plow, E. F., Frelinger, A. L., III, O'Toole, T. E., Loftus, J. C., & Ginsberg, M. H. (1991) *Cell 65*, 409−416.

Engvall, E., & Ruoslahti, E. (1977) *Int. J. Cancer 20*, 1−5.

Frelinger, A. L., III, Du, X. P., Plow, E. F., & Ginsberg, M. H. (1991) *J. Biol. Chem. 266*, 17106−17111.

Gailit, J., & Ruoslahti, E. (1988) *J. Biol. Chem. 263*, 12927−12932.

Gulino, D., Ryckewaert, J. J., Andrieux, A., Rabiet, M. J., & Marguerie, G. (1990) *J. Biol. Chem. 265*, 9575−9581.

Hartfield, P. J., Greaves, M. W., & Camp, R. D. R. (1993) *Biochem. Biophys. Res. Commun. 196*, 1183−1187.

Hendey, B., & Maxfield, F. R. (1993) *Blood Cells 19*, 143−164.

Hendey, B., Klee, C. B., & Maxfield, F. R. (1992) *Science 258*, 296−299.

Hermanowski-Vosatka, A., Van Strijp, J. A. G., Swiggard, W. J., & Wright, S. D. (1992) *Cell 68*, 341−352.

Hibbs, M. L., Xu, H., Stacker, S. A., & Springer, T. A. (1991a) *Science 251*, 1611−1613.

Hibbs, M. L., Jakes, S., Stacker, S. A., Wallace, R. W., & Springer, T. A. (1991b) *J. Exp. Med. 174*, 1227−1238.

Hillery, C. A., Smyth, S. S., & Parise, L. V. (1991) *J. Biol. Chem. 266*, 14663−14669.

Horwitz, A. F., Duggan, K., Buck, C. A., Beckerle, M. C., & Burridge, K. (1986) *Nature 320*, 531−533.

Hynes, R. O. (1992) *Cell 69*, 11−25.

Ingebritsen, T. S., Stewart, A. A., & Cohen, P. (1983) *Eur. J. Biochem. 132*, 297−307.

Kirchhofer, D., Gailit, J., Ruoslahti, E., Grzesiak, J., & Pierschbacher, M. D. (1990) *J. Biol. Chem. 265*, 18525−18530.

Kirchhofer, D., Grzesiak, J., & Pierschbacher, M. D. (1991) *J. Biol. Chem. 266*, 4471−4477.

Kouns, W. C., Wall, C. D., White, M. M., Fox, C. F., & Jennings, L. K. (1990) *J. Biol. Chem. 265*, 20594−20601.

LaFlamme, S. E., Akiyama, S. K., & Yamada, K. M. (1992) *J. Cell Biol. 117*, 437−447.

Leung-Hagesteijn, C. Y., Milankov, K., Michalak, M., Wilkins, J., & Dedhar, S. (1994) *J. Cell Sci. 107*, 589−600.

Lindberg, F. P., Gresham, H. D., Schwarz, E., & Brown, E. J. (1993) *J. Cell Biol. 123*, 485−496.

Marie, C., Tranqui, L., Soyez, S., & Block, M. R. (1991) *Exp. Cell Res. 192*, 173−181.

Marks, P. W., & Maxfield, F. R. (1990) *J. Cell Biol. 110*, 43−52.

Marks, P. W., Hendey, B., & Maxfield, F. R. (1991) *J. Cell Biol. 112*, 149−158.

Martin-Pérez, J., Rudkin, B. B., Siegmann, M., & Thomas, G. (1986) *EMBO J. 5*, 725−731.

Melançon, P., Glick, B. S., Malhotra, V., Weidman, P. J., Serafini, T., Gleason, M. L., Orci, L., & Rothman, J. E. (1987) *Cell 51*, 1053−1062.

Nairn, A. C., Hemmings, H. C., Jr., & Greengard, P. (1985) *Annu. Rev. Biochem. 54*, 931−976.

Neugebauer, K. M., & Reichardt, L. F. (1991) *Nature 350*, 68−71.

Otey, C. A., Pavalko, F. M., & Burridge, K. (1990) *J. Cell Biol. 111*, 721−729.

O'Toole, T. E., Loftus, J. C., Du, X., Glass, A. A., Ruggeri, Z. M., Shattil, S. J., Plow, E. F., & Ginsberg, M. H. (1990) *Cell Regul. 1*, 883−893.

O'Toole, T. E., Mandelman, D., Forsyth, J., Shattil, S. J., Plow, E. F., & Ginsberg, M. H. (1991) *Science 254*, 845−847.

Rojiani, M. V., Finlay, B. B., Gray, V., & Dedhar, S. (1991) *Biochemistry 30*, 9859−9866.

Schwartz, M. A., Brown, E. J., & Fazeli, B. (1993) *J. Biol. Chem. 268*, 19931−19934.

Shattil, S. J., & Brugge, J. S. (1991) *Curr. Opin. Cell Biol. 3*, 869−879.

Shaw, L. M., Lotz, M. M., & Mercurio, A. M. (1993) *J. Biol. Chem. 268*, 11401−11408.

Shenolikar, S., & Nairn, A. C. (1991) in *Advances in Second Messenger and Phosphoprotein Research* (Greengard, P., & Robison, G. A., Eds.) pp 1−121, Raven Press, New York, NY.

Shimizu, Y., Van Seventer, G. A., Horgan, K. J., & Shaw, S. (1990) *Nature 345*, 250−253.

Wilkins, J. A., Stupack, D., Stewart, S., & Caixia, S. (1991) *Eur. J. Immunol. 21*, 517−522.

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