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Integrin signaling: roles for the cytoplasmic tails of $\alpha_{IIb}\beta_3$ in the tyrosine phosphorylation of pp125^{FAK}

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SUMMARY

pp125^{FAK} (focal adhesion kinase) a protein tyrosine kinase that may mediate cellular responses to adhesion, is activated and tyrosine-phosphorylated when platelets adhere to fibrinogen via the integrin, $\alpha_{IIb}\beta_3$. To determine whether either of the cytoplasmic tails of $\alpha_{IIb}\beta_3$ regulates FAK phosphorylation, CHO cells were stably transfected with $\alpha_{IIb}\beta_3$ or various cytoplasmic tail truncation mutants. Cells expressing wild-type $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\beta_3$ that lacked the COOH-terminal 13 or 18 residues of the 20 residue α_{IIb} tail adhered to and spread on fibrinogen or on an anti-α_{IIb} antibody, and FAK became tyrosine-phosphorylated. FAK also became phosphorylated in adherent cells lacking the COOH-terminal 35 or 39 residues of the 47 residue β_3 tail, although the extent of phosphorylation was reduced by about 50% in the latter mutant. Little or no FAK phosphorylation was observed if 46 residues were deleted from the β_3 tail. None of these β_3 truncation mutants spread on the anti-α_{IIb} antibody. When cells with wild-type $\alpha_{IIb}\beta_3$ or truncated β_3 were detached from a surface, FAK became rapidly dephosphorylated. In contrast, FAK remained phosphorylated in the two α_{IIb} truncation mutants for up to 90 minutes in suspension. This persistent phosphorylation was not due to occupancy of $\alpha_{IIb}\beta_3$ by adhesive ligands because it was also observed with an α_{IIb} tail truncation mutant that contained an additional mutation in the extracellular portion of the receptor that prevents ligand binding. These studies demonstrate that: (1) the β_3 cytoplasmic tail, including the membrane-proximal portion, is involved in initiation of FAK phosphorylation; (2) FAK phosphorylation can be initiated by cell adhesion in the absence of cell spreading; and (3) the membrane-distal portion of the α_{IIb} cytoplasmic tail may normally function to dampen FAK phosphorylation in non-anchored cells.

Key words: integrin $\alpha_{IIb}\beta_3$, focal adhesion kinase, tyrosine phosphorylation

INTRODUCTION

Integrins are cell adhesion receptors, each composed of an α and a β type I transmembrane subunit. The subunits have a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail generally consisting of 20-70 amino acids (Hynes, 1992; Sastry and Horwitz, 1993). The platelet-specific integrin, $\alpha_{IIb}\beta_3$, mediates platelet aggregation and spreading on extracellular matrices through interactions with fibrinogen and von Willebrand factor (Weiss et al., 1989; Haimovich et al., 1993). These interactions are regulated such that high affinity binding of soluble ligands requires platelet activation (Shattil, 1995). Similarly, resting platelets do not adhere to immobilized von Willebrand factor and they adhere only loosely to fibrinogen, whereas activated platelets adhere tightly to both ligands (Haimovich et al., 1993). This mode of regulation, referred to as inside-out signaling, may involve interactions of the α_{IIb} and/or β_3 cytoplasmic tails with as yet uncharacterized intracellular mediators (Shattil, 1995; O'Toole et al., 1994).

$\alpha_{IIb}\beta_3$ and other integrins also participate in outside-in signaling whereby extracellular biochemical and mechanical cues are transduced into the cell. One of the earliest detectable integrin signaling events in platelets and other cells is protein tyrosine phosphorylation (Clark and Brugge, 1995). Of particular interest in this regard, the protein tyrosine kinase, pp125^{FAK} (FAK), is localized to integrin-rich focal adhesions in adherent cells and becomes activated and phosphorylated on tyrosine residues following ligand-induced integrin clustering (Hanks et al., 1992; Schaller and Parsons, 1994). Integrin ligation also triggers cytoskeletal reorganization and can influence programs of gene expression during cell growth, differentiation and programmed death (Juliano and Haskill, 1993; Roskelley et al., 1994; Meredith et al., 1993). FAK may function as a key mediator in these events by integrating signals from integrins with those from receptor tyrosine kinases and other plasma membrane receptors. Indeed, recent studies have identified regions or specific residues within FAK that target it to focal adhesions (Hildebrand et al., 1993; Schaller et al., 1995) or are responsible for its interactions with integrins (Schaller and Parsons, 1994), other kinases (Src, PI 3-kinase) (Cobb et al., 1994; Chen and Guan, 1994), and

adaptor proteins (Grb2, paxillin) (Turner and Miller, 1994; Schlaepfer et al., 1994).

Fibrinogen-dependent platelet adhesion and aggregation are accompanied by the phosphorylation of specific proteins on tyrosine residues (Ferrell and Martin, 1989; Golden et al., 1990). Some of these substrates, including a protein tyrosine kinase, pp72^{syk}, are phosphorylated within seconds of fibrinogen binding to $\alpha_{IIb}\beta_3$ (Huang et al., 1993; Clark et al., 1994). Others, including pp60^{Src} and FAK, are phosphorylated later during platelet aggregation or spreading (Huang et al., 1993; Haimovich et al., 1993), events that are associated with a major reorganization of the actin cytoskeleton (Hartwig, 1992; Fox et al., 1993). While sequences within the cytoplasmic tails of α_{IIb} and β_3 have been shown to influence certain aspects of outside-in signaling, such as integrin recruitment to focal adhesions (Ylanne et al., 1993, 1995), the function of the tails in initiating the tyrosine phosphorylation cascade in platelets has not been defined.

One approach to study the process of outside-in signaling through $\alpha_{IIb}\beta_3$ would be to delete portions of the cytoplasmic tails by mutagenesis. While this cannot be done in platelets, it can be done in Chinese hamster ovary (CHO) cells that have been stably transfected with $\alpha_{IIb}\beta_3$ (O'Toole et al., 1994). Therefore, to study the roles of the α_{IIb} and β_3 cytoplasmic tails in FAK phosphorylation, we have expressed $\alpha_{IIb}\beta_3$ mutants containing partial or complete deletions of the cytoplasmic tails in CHO cells. Tyrosine phosphorylation of FAK was examined in response to cell adhesion to immobilized $\alpha_{IIb}\beta_3$ ligands. The results show that both the α_{IIb} and β_3 cytoplasmic tails are involved in the regulation of FAK. Furthermore, under certain experimental conditions, FAK phosphorylation can occur in the absence of cell spreading, suggesting that full reorganization of the cytoskeleton is not required for initial activation of FAK.

MATERIALS AND METHODS

Production and characterization of CHO cell lines expressing human $\alpha_{IIb}\beta_3$

CHO cells were stably-transfected with various $\alpha_{IIb}\beta_3$ constructs and grown in the presence of fetal calf serum as described (O'Toole et al., 1990, 1991, 1994; Ylanne et al., 1993). Cell surface expression of $\alpha_{IIb}\beta_3$ was quantitated by flow cytometry, using a murine monoclonal antibody (Ab D57) specific for the $\alpha_{IIb}\beta_3$ complex (O'Toole et al., 1994).

Studies of FAK phosphorylation in adherent and suspended CHO cells

Tissue culture plates (100 mm; Falcon) were coated overnight at 4°C with one of the following proteins in coating buffer (150 mM NaCl, 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, pH 8): 5 mg/ml bovine serum albumin (BSA; fraction V, Sigma), 100 μg/ml purified human fibrinogen (Ugarova et al., 1993), 10 μg/ml murine myeloma protein IgG_1 (MOPC-21, Sigma), 10 μg/ml Ab PM1.1 (an anti-α_{IIb} monoclonal antibody; Ginsberg et al., 1986), 10 μg/ml Ab D57 (an anti-α_{IIb} monoclonal antibody), 10 μg/ml 7G7B6 (an anti-IL2 receptor monoclonal antibody; Chen et al., 1994), or 5 μg/ml poly-L-lysine (Sigma). The plates were then washed twice with phosphate-buffered saline (PBS), blocked for 2 hours at 37°C with 5 mg/ml BSA and then washed twice more with PBS.

CHO cells were trypsinized, washed once and resuspended to 1×10^7/ml in an incubation buffer containing 137 mM NaCl, 2.7 mM $MgCl_2$, 5.6 mM glucose, 3.3 mM NaH_2PO_4 and 20 mM Hepes, pH 7.4. Then 1 ml of cells was added to each protein-coated plate for 90 minutes at 37°C. Non-adherent cells were diluted 1:1 with PBS, sedimented at 100 ***g*** for 5 minutes, rinsed twice more with PBS, and lysed with RIPA buffer (158 mM NaCl, 1 mM Na_2EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 10 mM Tris-HCl, pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 (Fisher), and 100 KIU/ml aprotinin (Boehringer-Mannheim). Adherent cells were rinsed three times with PBS and either photographed by phase microscopy or scraped into RIPA buffer containing inhibitors. Lysates were clarified by centrifugation in a microfuge at 14,000 rpm at 4°C for 30 minutes and protein content was determined with the BCA reagent (Pierce).

Immunoprecipitation and western blotting of pp125^{FAK}

Equal amounts of protein from each lysate (250-500 μg, depending on the experiment) were incubated overnight at 4°C with either an affinity-purified rabbit polyclonal anti-phosphotyrosine antibody (UP28) (a gift from Joan Brugge, Ariad Pharmaceuticals, Inc., Cambridge, MA) (Huang et al., 1993), a rabbit antiserum specific for pp125^{FAK} (BC3, a gift from J. Thomas Parsons, University of Virginia, Charlottesville, VA) (Lipfert et al., 1992), or an appropriate control antibody. Immune complexes were precipitated at 4°C with Protein A-Sepharose that had been previously blocked with 10 mg/ml BSA for 10 minutes, followed by 3 washes in ice-cold RIPA buffer containing 1 mM Na_3VO_4. Immune complexes were extracted into boiling Laemmli sample buffer containing 10% β-mercaptoethanol and subjected to SDS-PAGE on 7.5% polyacrylamide gels and electrotransferred to nitrocellulose. Western blots were prepared and analyzed for phosphotyrosine-containing proteins as described previously (Huang et al., 1993), using the anti-phosphotyrosine antibody PY72 (a gift from Bart Sefton, Salk Institute, La Jolla, CA).

RESULTS

Interaction of $\alpha_{IIb}\beta_3$ with specific immobilized ligands stimulates tyrosine phosphorylation of pp125^{FAK} in CHO cells

Adhesion of platelets to immobilized fibrinogen requires surface expression of $\alpha_{IIb}\beta_3$. Over the course of 15-90 minutes, the adherent platelets spread and exhibit tyrosine phosphorylation of numerous proteins, including pp125^{FAK} (Haimovich et al., 1993). As with platelets, a stable CHO cell line (A5) that expresses human α_{IIb} and β_3 adheres to and spreads on fibrinogen, whereas untransfected cells do not (Ylanne et al., 1993). Therefore, to study the role of the cytoplasmic tails of α_{IIb} or β_3 in the tyrosine phosphorylation of FAK, human α_{IIb} and β_3 containing specific truncations of the tails were stably expressed in CHO cells.

First, it was necessary to prove that tyrosine phosphorylation of hamster FAK occurs in A5 cells in response to cell adhesion to an $\alpha_{IIb}\beta_3$ ligand. As expected, A5 cells attached to and spread on a fibrinogen-coated surface, but they did not adhere to a BSA-coated surface. Untransfected CHO cells did not bind to either surface. To study protein tyrosine phosphorylation, A5 cell lysates were immunoprecipitated with an anti-phosphotyrosine polyclonal antibody to, in effect, concentrate phosphotyrosine-containing proteins. The immunoprecipitates were then probed on western blots with a monoclonal anti-phosphotyrosine antibody. A5 cells suspended over BSA for 90 minutes at 37°C exhibited tyrosine phosphorylation of three prominent bands at ~40-50 kDa, 70-80 kDa and 120-130 kDa.

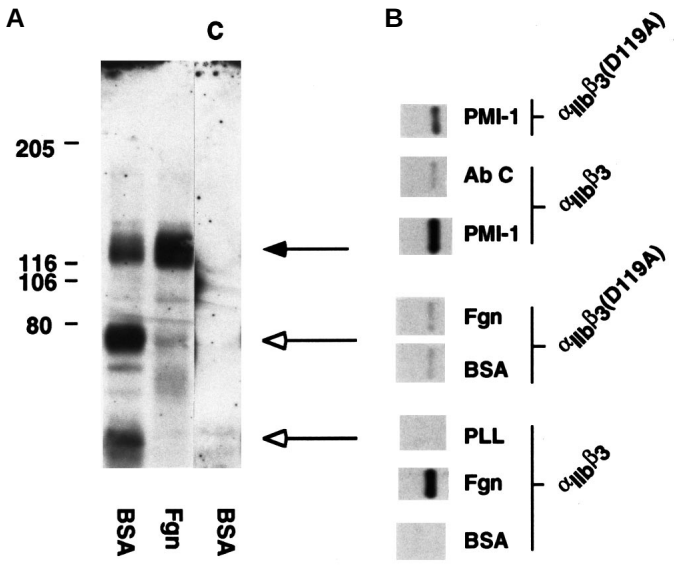

Fig. 1. Tyrosine phosphorylation of FAK and other substrates in CHO cells transfected with human $\alpha_{IIb}\beta_3$. (A) A5 cells stably-transfected with $\alpha_{IIb}\beta_3$ were added to plates coated with BSA or fibrinogen (Fgn) as described in Materials and Methods. After 90 minutes at 37°C, lysates (500 μg) of cells that were adherent to fibrinogen or not adherent to BSA were immunoprecipitated with either UP28, a rabbit antibody specific for phosphotyrosine, or normal rabbit serum (C). Immunoprecipitates were immunoblotted with antiphosphotyrosine antibody, PY72. The filled arrow points to the band at 120-130 kDa that increased in adherent cells, and the open arrows point to bands that decreased. Not shown is the fact that non-transfected CHO cells incubated over BSA or fibrinogen remained in suspension and showed the same phosphotyrosine profile as the A5 cells in suspension over BSA. (B) A5 cells or cells transfected with a ligand-binding defective form of the integrin, $\alpha_{IIb}\beta_3$(D119A), were incubated for 90 minutes at 37°C in plates coated with either BSA, Fgn, poly-L-lysine (PLL), mouse monoclonal Ab PM1.1 (specific for α_{IIb}) or control mouse IgG_1 myeloma protein (Ab C). A5 cells failed to bind to BSA or Ab C but bound to fibrinogen and Ab PM1.1; $\alpha_{IIb}\beta_3$(D119A) cells failed to bind to BSA or fibrinogen but bound to Ab PM1.1. Cell lysates were immunoprecipitated with the anti-FAK antiserum, BC3, and immunoblots were probed with PY72. The bands represent $pp125^{FAK}$.

In contrast, when the cells were allowed to attach to and spread on fibrinogen for 90 minutes, there was a marked *decrease* in intensity of the 40-50 kDa and 70-80 kDa bands and an *increase* in intensity of the 120-130 kDa band (Fig. 1A). Thus, adhesion of CHO cells via $\alpha_{IIb}\beta_3$ can exert differential effects on tyrosine kinase substrates, increasing the state of phosphorylation of some and decreasing others.

To establish if one of the tyrosine-phosphorylated proteins in the 120-130 kDa region of the blot shown in Fig. 1 represented FAK, parallel cell lysates were immunoprecipitated with a polyclonal anti-FAK antibody and western blots were probed with a monoclonal anti-phosphotyrosine antibody. No FAK phosphorylation was observed in A5 cells maintained in suspension for 90 minutes over a BSA-coated surface. In contrast, FAK became phosphorylated during cell adhesion and spreading on fibrinogen (Fig. 1B). Tyrosine phosphorylation was detectable at the earliest time point studied (15 minutes), was maximal by 60 minutes and remained steady for up to 90 minutes. Re-probing the blots with a monoclonal anti-FAK antibody showed that equal amounts of this protein had been immunoprecipitated from each sample, indicating that cell adhesion had increased the extent of FAK phosphorylation (not shown).

FAK phosphorylation in A5 cells required the specific interaction of $\alpha_{IIb}\beta_3$ with fibrinogen because: (1) A5 cells adherent to a non-specific substrate, poly-L-lysine, exhibited no tyrosine phosphorylation of FAK; (2) cells expressing a point mutation in the extracellular portion of the β_3 subunit (D119A) that abolishes ligand binding (Loftus et al., 1990) failed to adhere to fibrinogen or exhibit FAK phosphorylation; and (3) FAK became phosphorylated in cells expressing either $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\beta_3$(D119A) when they were allowed to attach to Ab PM1.1, a non-function-blocking antibody against α_{IIb} (Fig. 1B). Thus, the adhesion of CHO cells to fibrinogen is dependent on $\alpha_{IIb}\beta_3$ and leads to tyrosine phosphorylation of FAK.

Effect of truncations of the β_3 cytoplasmic tail on FAK phosphorylation

Clustering of β_1, β_3 or β_5 integrin cytoplasmic tail chimeras in fibroblasts is sufficient to cause tyrosine phosphorylation of FAK (Akiyama et al., 1994; Lukashev et al., 1994). To examine the requirement for the β_3 tail in the context of an intact integrin, wild-type α_{IIb} was stably expressed in CHO cells along with one of three truncated forms of β_3: $\beta_3\Delta728$, $\beta_3\Delta724$ or $\beta_3\Delta717$. $\beta_3\Delta728$ lacks the COOH-terminal 35 residues of the 47 residue β_3 tail, $\beta_3\Delta724$ lacks the COOH-terminal 39 residues and $\beta_3\Delta717$ lacks the COOH-terminal 46 residues (Table 1). All variants of $\alpha_{IIb}\beta_3$ were expressed on the surface of the CHO cells, although expression of $\alpha_{IIb}\beta_3\Delta728$ was typically somewhat greater and $\alpha_{IIb}\beta_3\Delta717$ typically less than that of $\alpha_{IIb}\beta_3$ (Fig. 2). Unlike A5 cells, $\alpha_{IIb}\beta_3\Delta728$ and $\alpha_{IIb}\beta_3\Delta724$ cells failed to spread (Fig. 3) or to form focal

Table 1. Sequences of the cytoplasmic tails of αIIb, β3 and truncation mutants

α_{IIb}	^{989}KVGFFKRNRPPLEEDDEEGE*
$\alpha_{IIb}\Delta996$	^{989}KVGFFKR
$\alpha_{IIb}\Delta991$	^{989}KV
β_3	^{716}KLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT
$\beta_3\Delta728$	^{716}KLLITIHDRKEF
$\beta_3\Delta724$	^{716}KLLITIHD
$\beta_3\Delta717$	^{716}K

*The numbers of the first lysine residue in each sequence denotes the number of that amino acid in the full-length integrin sequence. These lysine residues are assumed to represent the exit point of the cytoplasmic tails from the plasma membrane (Williams et al., 1994).

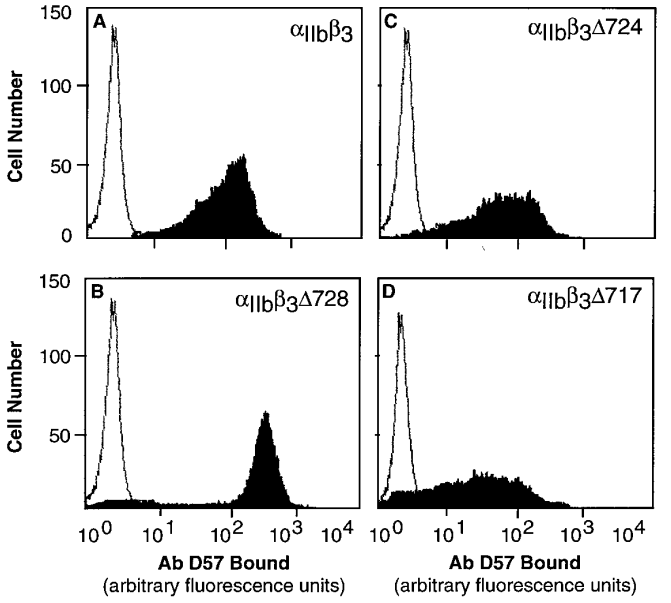

Fig. 2. $\alpha_{IIb}\beta_3$ expression in CHO cells transfected with (A) wild-type $\alpha_{IIb}\beta_3$, (B) $\alpha_{IIb}\beta_3\Delta728$, (C) $\alpha_{IIb}\beta_3\Delta724$ or (D) $\alpha_{IIb}\beta_3\Delta717$. Surface expression was determined by flow cytometry using the $\alpha_{IIb}\beta_3$-specific antibody, D57. Data for 10,000 cells were obtained by gating on live cells identified by light scatter. Ab D57 binding is expressed in arbitrary fluorescence units. This experiment is representative of three so performed.

adhesions (Ylanne et al., 1993) during cell adhesion to immobilized fibrinogen or to Ab PM1.1 and D57, antibodies specific for α_{IIb} and $\alpha_{IIb}\beta_3$, respectively. Similarly, $\alpha_{IIb}\beta_3\Delta717$ cells did not spread on these antibodies, but some cells did assume a partially-spread orientation on fibrinogen (Fig. 3).

Tyrosine phosphorylation of FAK was studied both in the presence and absence of 20 μM cycloheximide. This compound was used in an attempt to prevent the de novo synthesis of fibronectin or other adhesive ligands by the CHO cells and thus to minimize induction of FAK phosphorylation by ligation of hamster integrins. Cycloheximide was not generally toxic to CHO cells, since all of these cell lines exhibited tyrosine phosphorylation of FAK upon adhesion to purified fibronectin, presumably via hamster β_1 integrins (Fig. 4). Concentrations of cycloheximide in this range have been used for similar purposes by others (Schultz and Arman, 1995).

When each of the three β_3 truncation mutants were plated over BSA in the presence of cycloheximide, they did not adhere and no FAK phosphorylation was observed. However, as shown for a single experiment in Fig. 4, FAK phosphorylation was observed in A5 cells after adhesion to the $\alpha_{IIb}\beta_3$ antibody, Ab D57. FAK phosphorylation was also detectable in $\alpha_{IIb}\beta_3\Delta728$ and in $\alpha_{IIb}\beta_3\Delta724$ cells adherent to Ab D57, but less so in $\alpha_{IIb}\beta_3\Delta717$ cells (Fig. 4). Densitometric analyses of western blots from 3 such experiments were carried out to quantitate the extent of FAK phosphorylation triggered by cell adhesion to the $\alpha_{IIb}\beta_3$ antibody. Compared to A5 cells, FAK phosphorylation in $\alpha_{IIb}\beta_3\Delta728$ cells was reduced by an average of only 4.4%, while phosphorylation of FAK in $\alpha_{IIb}\beta_3\Delta724$ and $\alpha_{IIb}\beta_3\Delta717$ cells was reduced by 46.7% and 71.1%, respectively (Fig. 5). Parallel immunoblots probed with an anti-FAK antibody demonstrated that equal amounts of FAK had been immunoprecipitated from each of these cell lines (not shown). The residual FAK phosphorylation in the $\alpha_{IIb}\beta_3\Delta717$ cells observed in some experiments could not be explained by expression of the β_3 cytoplasmic tail because $\alpha_{IIb}\beta_3$ immunoprecipitated from $\alpha_{IIb}\beta_3\Delta717$ cell lysates failed to react on western blots with a polyclonal antibody specific for the β_3 tail. Two conclusions can be drawn from these experiments. First, FAK phosphorylation triggered through $\alpha_{IIb}\beta_3$ occurs in the absence of distal sequences of the β_3 cytoplasmic tail. Second, since the $\alpha_{IIb}\beta_3\Delta728$ and $\alpha_{IIb}\beta_3\Delta724$ cells failed to spread on fibrinogen yet exhibited FAK phosphorylation, initiation of FAK phosphorylation in these CHO cell lines is not dependent on cell spreading.

Effect of truncations of the α_{IIb} cytoplasmic tail on FAK phosphorylation

To examine the role of the α_{IIb} cytoplasmic tail in integrin signaling, wild-type β_3 was expressed in CHO cells along with one of two truncated forms of α_{IIb}. $\alpha_{IIb}\Delta996$ lacks the carboxy-terminal 13 residues of the 20 residue α_{IIb} cytoplasmic tail, but it preserves the highly conserved membrane-proximal KVGFFKR segment. $\alpha_{IIb}\Delta991$ lacks the carboxy-terminal 18 residues of the cytoplasmic tail (Table 1). Previous studies have shown that, like wild-type $\alpha_{IIb}\beta_3$, $\alpha_{IIb}\Delta996\beta_3$ exists in a low-affinity state in CHO cells with respect to soluble ligands, while $\alpha_{IIb}\Delta991\beta_3$ exists in a high affinity state (O'Toole et al., 1994). Both mutant integrins differ from $\alpha_{IIb}\beta_3$ in that they are recruited to focal adhesions in a ligand-independent manner (Ylanne et al., 1993). As shown in Fig. 6 for $\alpha_{IIb}\Delta996\beta_3$, these integrins were expressed on the cell surface to levels of about 50% of that observed for $\alpha_{IIb}\beta_3$ in A5 cells. Nonetheless, when the mutant cells were incubated for 90 minutes over a fibrinogen matrix, they became adherent and fully spread (not shown) and they exhibited tyrosine phosphorylation of FAK (Fig. 7). Thus, the α_{IIb} cytoplasmic tail is not necessary for FAK phosphorylation in response to cell adhesion via $\alpha_{IIb}\beta_3$. This is consistent with a previous study of $\alpha_5\beta_1$ in fibronectin-adherent CHO cells that concluded that the α_5 cytoplasmic domain is not essential for integrin-mediated tyrosine phosphorylation (Bauer et al., 1993).

However, studies of these α_{IIb} mutant cells in suspension indicated that the α_{IIb} cytoplasmic tail can *modulate* the process of FAK tyrosine phosphorylation. When A5 cells were removed from tissue culture plastic and incubated in suspension, FAK phosphorylation became undetectable within five minutes and remained so for up to 90 minutes. In contrast, when $\alpha_{IIb}\Delta996\beta_3$ cells were maintained in suspension, FAK remained phosphorylated on tyrosine residues for up to 90 minutes (Fig. 7). Identical results were obtained with the $\alpha_{IIb}\Delta991$ cell line (not shown). Although the extent of residual FAK phosphorylation varied from experiment to experiment, this basic observation held true for multiple clonal isolates of each cell line. The presence of the β_3 tail was necessary for persistent FAK phosphorylation in the suspended α_{IIb} mutant cells because it was not observed in an α_{IIb} mutant cell line that also lacks the β_3 tail ($\alpha_{IIb}\Delta996\beta_3\Delta717$) (not shown).

Four separate observations indicated that the persistent phosphorylation of FAK in the suspended cells did not require occupancy of the α_{IIb} mutant integrins by adhesive ligands. First, no soluble ligand was added to the suspended cells.

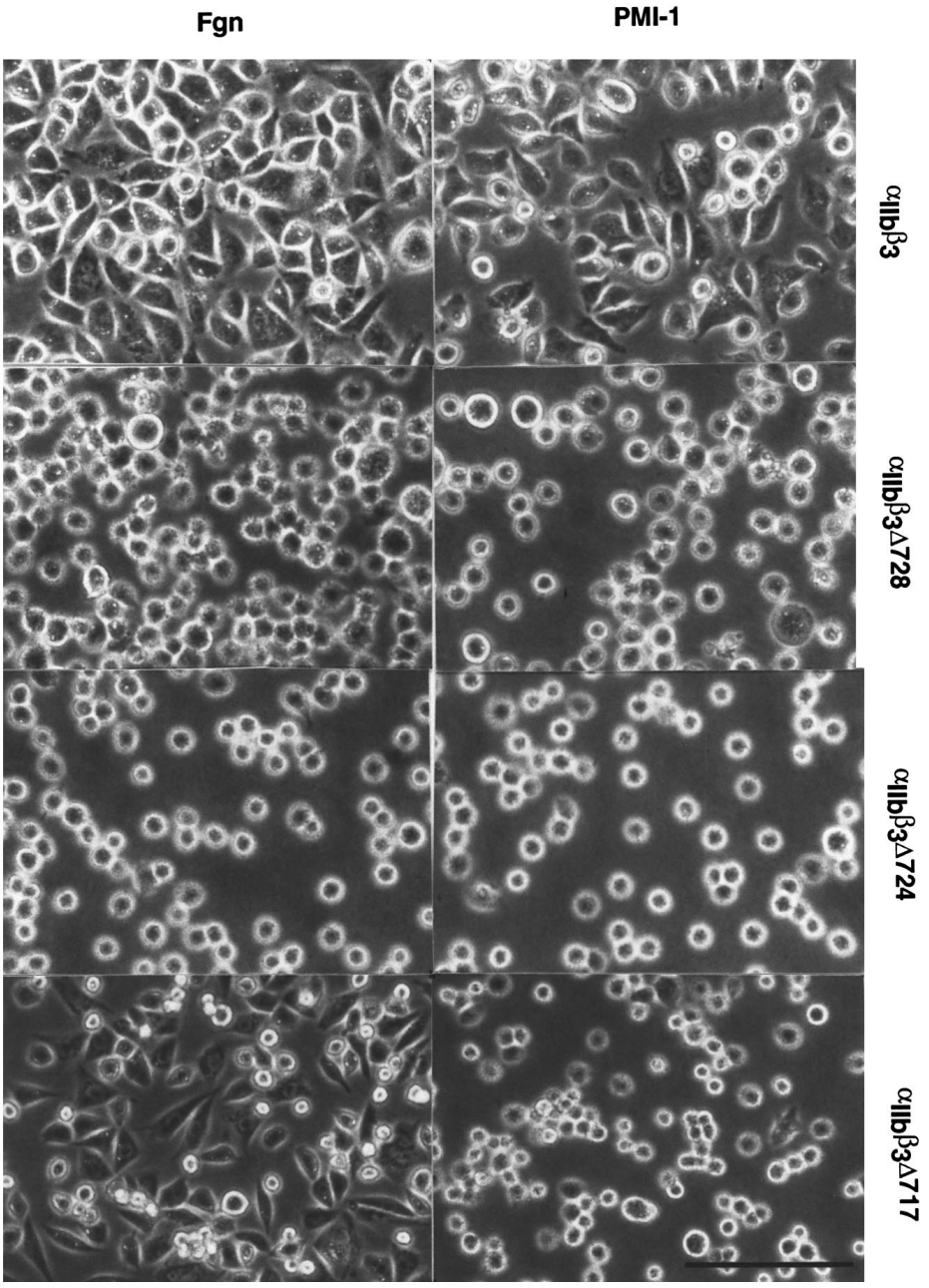

Fig. 3. Truncation of the β_3 cytoplasmic tail results in decreased spreading of $\alpha_{IIb}\beta_3$-bearing CHO cells adherent to $\alpha_{IIb}\beta_3$ matrices. Transfectants were incubated in plates coated with fibrinogen or Ab PM1.1 for 90 minutes at 37°C. After removal of non-adherent cells, adherent cells were washed twice with PBS, and photographed under a phase-contrast microscope (×400; bar, 100 μm).

Second, the $\alpha_{IIb}\Delta996\beta_3$ mutant is in a low affinity state and would not be expected to bind a soluble ligand. Third, addition of 20 μM cycloheximide to minimize endogenous synthesis of a ligand had no effect on FAK phosphorylation in the α_{IIb} mutants. Fourth, persistent tyrosine phosphorylation of FAK was still observed in suspensions of a stable cell line that expressed a double mutation, $\alpha_{IIb}\Delta996\beta_3$(D119A) (Fig. 7). The β_3(D119A) mutation is in the extracellular domain of β_3 and prevents ligand binding (Loftus et al., 1990). Taken together, these experiments show that removal of at least two-thirds of the residues from the COOH terminus of the α_{IIb} cytoplasmic tail leads to anomalous tyrosine phosphorylation of FAK in suspended CHO cells bearing $\alpha_{IIb}\beta_3$. This suggests that the α_{IIb} tail may normally function to dampen integrin signaling when cells are not adherent to a surface.

DISCUSSION

Characterization of a heterologous expression system to study integrin signaling

In several cell types, ligand binding to and clustering of integrins is associated with tyrosine phosphorylation and activation of FAK and localization of the enzyme to focal adhesions (Guan et al., 1991; Kornberg et al., 1991; Hanks et al., 1992; Lipfert et al., 1992; Hildebrand et al., 1993). Membrane-based focal adhesions or closely-related structures form in many types of adherent cells, including platelets (Nachmias and Golla, 1991), and they couple extracellular matrix ligands to actin stress fibres through integrins (Turner and Burridge, 1991). Since focal adhesions contain numerous enzymes (e.g. pp60^{Src}, PI 3-kinase, protein kinase C) as well

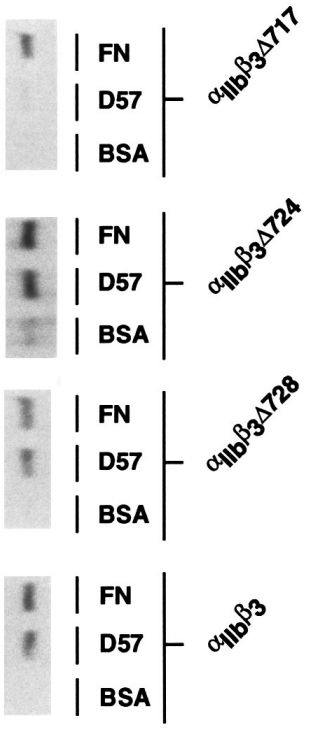

Fig. 4. Effects of truncations of the β_3 cytoplasmic tail on FAK phosphorylation in $\alpha_{IIb}\beta_3$-bearing CHO cells. Cells transfected with either wild-type $\alpha_{IIb}\beta_3$, $\alpha_{IIb}\beta_3\Delta728$, $\alpha_{IIb}\beta_3\Delta724$ or $\alpha_{IIb}\beta_3\Delta717$ were incubated for 1 hour at 37°C in tissue culture medium in the presence of 20 μM cycloheximide. Cells were then detached from the tissue culture plates with trypsin-EDTA, pelleted and resuspended in incubation buffer in the presence of cycloheximide, and incubated for 90 minutes at 37°C in BSA-, Ab D57- or fibronectin (FN)-coated plates. Cells were then washed, lysed and 250 μg of protein were processed to assess FAK tyrosine phosphorylation. This experiment is representative of three so performed.

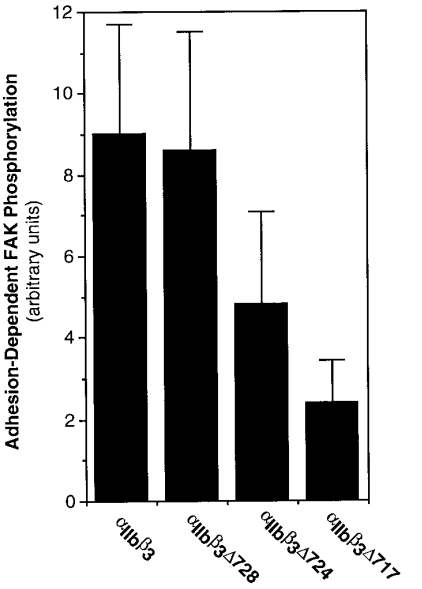

Fig. 5. Tyrosine phosphorylation of FAK in various CHO cell lines adherent to the anti-$\alpha_{IIb}\beta_3$ antibody, Ab D57. The experimental conditions were as in Fig. 4. The extent of FAK phosphorylation was quantitated by scanning luminograms on a flatbed scanner and performing calibrated densitometric analysis with the program, NIH Image 1.53. Densitometric values for FAK phosphorylation triggered by cell adhesion were obtained by subtracting baseline values observed in non-adherent cells incubated over BSA for 90 minutes from the values obtained in cells adherent to Ab D57 for 90 minutes. Data are expressed in arbitrary units and represent means ± s.e.m. of three experiments. Compared to cells expressing $\alpha_{IIb}\beta_3$, the reduction in adhesion-dependent FAK phosphorylation was statistically significantly for the $\alpha_{IIb}\beta_3\Delta717$ cells ($P<0.04$, paired *t*-test) but not for the other two β_3 truncation mutants.

as cytoskeletal proteins (e.g. talin, α-actinin, paxillin), they may function as a type of 'signaling organelle' to facilitate information flow from the plasma membrane to the nucleus (Turner and Burridge, 1991; Cobb et al., 1994; Chen and Guan, 1994; Woods and Couchman, 1992; Sastry and Horwitz, 1993). Integrins are presumed to interact with other focal adhesion components through their cytoplasmic tails. Consistent with a role for these tails in integrin signaling, clustering of integrin β_1, β_3 or β_5 cytoplasmic tail chimeras in fibroblasts is sufficient to effect tyrosine phosphorylation of FAK (Akiyama et al., 1994; Lukashev et al., 1994).

To study the signaling function of integrin tails in more detail, we have expressed $\alpha_{IIb}\beta_3$ in CHO cells. The effect of deletions of cytoplasmic tail sequences on tyrosine phosphorylation of hamster FAK was studied by plating the cells on matrices selective or specific for $\alpha_{IIb}\beta_3$. This experimental system was validated by showing that transfectants expressing wild-type $\alpha_{IIb}\beta_3$ failed to adhere to a BSA-coated matrix or to exhibit tyrosine phosphorylation of FAK, and no phosphorylation occurred during 'non-specific' cell attachment to poly-L-lysine. However, when cells were incubated either over a fibrinogen matrix or anti-$\alpha_{IIb}\beta_3$ monoclonal antibodies, they attached and spread and tyrosine phosphorylation of FAK was observed. It is interesting to note that certain phosphotyrosine-containing proteins became *dephosphorylated* during CHO cell adhesion to fibrinogen, consistent with observations in platelets that adhesion via $\alpha_{IIb}\beta_3$ stimulates both protein tyrosine kinases and phosphatases (Frangione et al., 1993; Luber and Siess, 1994; Ezumi et al., 1995). Thus, although the current studies were focused on FAK, the CHO cell system may also prove useful in studying the functions of tyrosine phosphatases during integrin signaling.

Three major conclusions can be drawn from the present studies: (1) The distal β_3 tail is not necessary for integrin-triggered tyrosine phosphorylation of FAK. (2) Although the α_{IIb} tail is not necessary for FAK phosphorylation, sequences within the membrane-distal portion of the α_{IIb} tail can modulate this process. (3) Under certain experimental conditions, tyrosine phosphorylation of FAK does not require spreading of CHO cells on the adhesive matrix. As discussed later, this finding has implications for the relationship of FAK function to cytoskeletal reorganization.

The β_3 cytoplasmic tail and FAK phosphorylation

Tyrosine phosphorylation of FAK was reduced in CHO cells adherent through a form of $\alpha_{IIb}\beta_3$ that lacks the COOH-terminal 46 residues from the β_3 tail. This is consistent with previous studies using different experimental approaches indi-

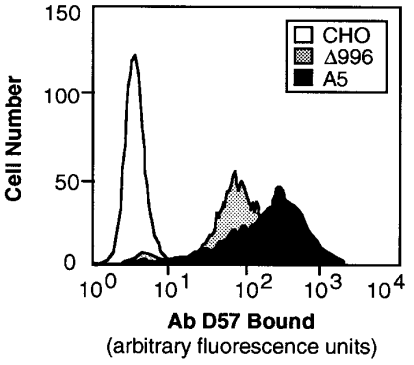

Fig. 6. Surface expression of $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}\Delta996\beta_3$ in stably-transfected CHO cell lines. Flow cytometric data were obtained as described in the legend to Fig. 2 and are representative of 3 experiments so performed.

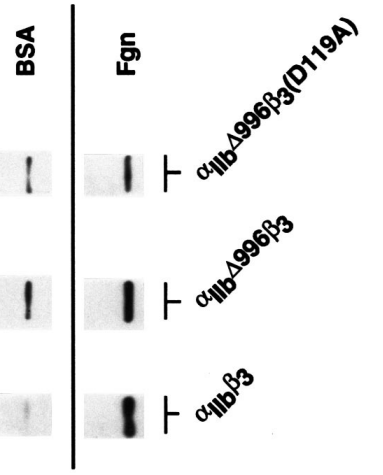

Fig. 7. Effects of truncations of the α_{IIb} cytoplasmic tail on FAK phosphorylation in $\alpha_{IIb}\beta_3$-bearing CHO cells. The indicated cell lines were incubated for 90 minutes at 37°C in either BSA- or fibrinogen-coated plates. The cells failed to adhere to the BSA plates but adhered to the fibrinogen plates. Non-adherent cells in the BSA plates and the adherent cells in the fibrinogen plates were processed for analysis of FAK tyrosine phosphorylation as described in Materials and Methods. This experiment is representative of five so performed.

cating that a β tail is involved in integrin-mediated FAK phosphorylation (Guan et al., 1991; Akiyama et al., 1994; Lukashev et al., 1994). It should be noted, however, that reduced surface expression of $\alpha_{IIb}\beta_3(\Delta717)$ compared to $\alpha_{IIb}\beta_3$ (Fig. 2) might have played some role in the reduced FAK phosphorylation (Figs 4 and 5). On the other hand, FAK phosphorylation was compared in cells adherent to an $\alpha_{IIb}\beta_3$-specific subtrate. Consequently, differences in $\alpha_{IIb}\beta_3$ surface expression may not have been as marked in these adherent cells. FAK phosphorylation in adherent cells appeared to be initiated through the transfected and truncated integrin since an antibody specific for $\alpha_{IIb}\beta_3$ was used as the ligand, and this antibody did not support the adhesion of untransfected CHO cells. The residual FAK phosphorylation exhibited by adherent β_3 truncation mutants may have been due to a component of adhesion through endogenous hamster integrins, despite the presence of cycloheximide to inhibit matrix synthesis. These data suggest that sequences within the β_3 tail are required for maximal FAK phosphorylation. The findings in this study seem to differ from a previous report showing that deletion of only 4 residues from the COOH terminus of the β_1 tail abolished tyrosine phosphorylation of a ~120 kDa band in fibroblasts adherent to fibronectin (Guan et al., 1991). Although the reasons for these differences are not apparent, the two studies focused on different integrins and different cell types, and the previous study performed anti-phosphotyrosine immunoblots on total cell lysates rather than on FAK immunoprecipitates.

In theory, the β_3 tail could function as a direct binding site for FAK. Upon fibrinogen binding to $\alpha_{IIb}\beta_3$, integrin clustering might then lead to FAK clustering, a process that could lead to FAK auto-phosphorylation. Alternatively, the β_3 tail might serve as a nucleation site for other protein tyrosine kinases that in turn could phosphorylate and activate FAK. Both of these possibilities are plausible. In vitro, certain integrin β tails have been shown to bind to α-actinin and talin (Otey et al., 1993; Horwitz et al., 1986). In addition, synthetic peptides derived from the membrane-proximal 13 residues of β_1 and β_3 can bind to the unique NH_2-terminal domain of FAK (Schaller et al., 1995). However, it is not yet proven that these interactions actually take place within cells. Moreover, integrin clustering by fibrinogen or antibodies is not sufficient to initiate the phosphorylation of FAK in platelets. Rather, costimulation of platelets with excitatory agonists as well as platelet aggregation (or spreading) are required (Shattil et al., 1994). After fibrinogen binding to platelet $\alpha_{IIb}\beta_3$, activation of $pp72^{Syk}$ and $pp60^{Src}$ precedes activation of FAK (Clark et al., 1994). Thus, in platelets at least, events in addition to integrin clustering are necessary for FAK phosphorylation. Of potential importance in this regard, activated $pp60^{c\text{-}Src}$ localizes to focal adhesions through its SH2 and SH3 domains (Kaplan et al., 1994) where it may participate in the recruitment and phosphorylation of FAK (Cobb et al., 1994).

The reduced FAK phosphorylation observed with β_3 cytoplasmic tail truncation could be due to fewer numbers of FAK molecules phosphorylated per cell or to less phosphorylation of each FAK molecule. FAK contains a number of potential sites of tyrosine phosphorylation that might be differentially regulated during cell adhesion, leading to different routes of integrin signaling. For example, Tyr^{397} in the NH_2-terminal domain of FAK is the major site of autophosphorylation and serves as a docking site for the SH2 domain of $pp60^{Src}$ (Schaller and Parsons, 1994; Calalb et al., 1995). On the other hand, Tyr^{925} in the COOH-terminal domain may function as a docking site for the SH2 domain of Grb2, thereby linking integrin signaling to Ras pathways (Schlaepfer et al., 1994).

The α_{IIb} cytoplasmic tail and FAK phosphorylation

FAK was rapidly dephosphorylated on tyrosine residues when CHO cells expressing wild-type $\alpha_{IIb}\beta_3$ were suspended in physiological buffer. In contrast, the $\alpha_{IIb}\Delta996\beta_3$ cell line containing a deletion of all but the membrane-proximal 7 residues of the 20 residue α_{IIb} cytoplasmic tail showed persistent FAK phosphorylation when the cells were maintained in suspension for up to 90 minutes. Similar results were obtained with $\alpha_{IIb}\Delta991\beta_3$ cells lacking the COOH-terminal 18 residues from the α_{IIb} tail. These results could not be

explained by the binding of soluble ligands to these integrins because $\alpha_{IIb}\Delta 996\beta_3$ is in a low affinity state in CHO cells (O'Toole et al., 1994). Moreover, persistent FAK phosphorylation was observed in a double-mutant cell line, $\alpha_{IIb}\Delta 996\beta_3$(D119A), that is incapable of binding soluble $\alpha_{IIb}\beta_3$ ligands. It is not known if the persistent phosphorylation in the α_{IIb} mutants is due to continued activity of a tyrosine kinase and/or to reduced activity of a tyrosine phosphatase. While further studies will be required to assess the functional significance of this anomalous signaling, these findings suggest that the distal portion of the α_{IIb} tail may normally function to dampen integrin signaling when cells are not adherent to a matrix.

Circular dichroism measurements of synthetic peptides and fluorescence quenching studies of neoproteins chemically synthesized to simulate the $\alpha_{IIb}\beta_3$ tails suggest that the tails can interact with each other (Haas et al., 1993; Muir et al., 1994). If this is true inside of cells, the α_{IIb} tail may function in non-anchored cells to occlude binding sites on the β_3 tail for proteins that are involved in integrin signaling. In the absence of the α_{IIb} tail, signaling might therefore proceed in an anomalous fashion, e.g. when cells are in suspension. Similarly, when normal cells become adherent to an integrin ligand, these physical relationships between the α and β tails might be modified so as to promote association of the β_3 tail with FAK and other focal adhesion proteins. Consistent with this hypothesis, both $\alpha_{IIb}\Delta 996\beta_3$ and $\alpha_{IIb}\Delta 991\beta_3$ become recruited to focal adhesions in a ligand-independent fashion during CHO cell adhesion to fibronectin via hamster β_1 integrins. This recruitment does not take place in the absence of the β_3 tail (Ylanne et al., 1993).

Cytoskeletal reorganization and FAK phosphorylation

In platelets and other cells, FAK phosphorylation is usually associated with cell spreading or aggregation, responses that require actin polymerization and rearrangements of the cytoskeleton, including formation of F-actin stress fibres and assembly of focal adhesions. In some cell types in which careful time course studies have been performed, FAK phosphorylation precedes cell spreading, implying that FAK may play a role in the spreading process (Guan et al., 1991). The finding that a tyrosine kinase inhibitor prevents FAK phosphorylation and cell spreading in fibroblasts is consistent with this interpretation (Burridge et al., 1992). On the other hand, in mouse aortic smooth muscle cells adherent to fibronectin, FAK activation is not required for the assembly of F-actin stress fibres or focal adhesions (Wilson et al., 1995). It is also possible that cytoskeletal rearrangements may promote FAK phosphorylation and activation by recruiting this enzyme to the correct subcellular location. Indeed, cytochalasin B or D, compounds that disrupt actin filaments, also inhibit integrin-triggered FAK phosphorylation in platelets and fibroblasts (Lipfert et al., 1992; Sinnett-Smith et al., 1993; Zachary et al., 1993). Yet, in the present study, cells bearing β_3 truncation mutants underwent some degree of FAK phosphorylation despite the fact that they did not spread on an anti-α_{IIb} antibody. This indicates that complete cytoskeletal reorganization is not required for the initiation of FAK phosphorylation in these cells. These diverse observations indicate that the exact relationship between FAK activation and cytoskeletal reorganization during cell adhesion may depend on the particular matrix, integrin and cell type studied.

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