α1 Integrin cytoplasmic domain is involved in focal adhesion formation via association with intracellular proteins

Klemens LÖSTER, Dörte VOSSMEYER, Werner HOFMANN, Werner REUTTER and Kerstin DANKER[1]

Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, D-14195 Berlin-Dahlem, Germany

Integrins are heterodimeric adhesion receptors consisting of α- and β-subunits capable of binding extracellular matrix molecules as well as other adhesion receptors on neighbouring cells. These interactions induce various signal transduction pathways in many cell types, leading to cytoskeletal reorganization, phosphorylation and induction of gene expression. Integrin ligation leads to cytoplasmic protein–protein interactions requiring both integrin cytoplasmic domains, and these domains are initiation points for focal adhesion formation and subsequent signal transduction cascades. In previous studies we have shown that the very short cytoplasmic α1 tail is required for post-ligand events, such as cell spreading as well as actin stress-fibre formation. In the present paper we report that cells lacking the cytoplasmic domain of the α1 integrin subunit are unable to form proper focal adhesions and that phosphorylation on tyrosine residues of focal adhesion components is reduced on $\alpha1\beta1$-specific substrates. The α1 cytoplasmic sequence is a specific recognition site for focal adhesion components like paxillin, talin, α-actinin and pp125FAK. It seems to account for α1-specific signalling, since when peptides that mimic the cytoplasmic domain of α1 are transferred into cells, they influence $\alpha1\beta1$-specific adhesion, presumably by competing for binding partners. For α1 integrin/protein binding, the conserved Lys-Ile-Gly-Phe-Phe-Lys-Arg motif and, in particular, the two lysine residues, are important.

Key words: α-actinin, cytoplasmic deletion, focal adhesion kinase, paxillin, talin.

INTRODUCTION

Integrins, expressed on virtually every cell type, are transmembrane proteins that mediate binding to components of the extracellular matrix and to other cell adhesion molecules on other cells. In addition, integrins participate in cell migration, tissue organization, cell growth, haemostasis, inflammation, target recognition of lymphocytes and differentiation of many cell types. Upon interaction with ligands, integrins cluster and associate with a variety of cytoplasmic proteins to form focal adhesions and the actin cytoskeleton.

The cytoplasmic domains of integrins are required for the formation of focal adhesions and cytoskeletal structures. The β1 cytoplasmic domain binds to focal adhesion proteins, such as talin [1] and α-actinin [2]. More recent studies have mapped functionally important sequences of cytoplasmic domains of β integrin subunits which bind to specific cytoskeletal molecules, such as α-actinin, paxillin and talin, as well as to signalling molecules like focal adhesion kinase (pp125FAK) [3]. The β1 cytoplasmic domain is required for localization of recombinant $\alpha5\beta1$ to existing focal adhesions [4–6].

Initial binding of proteins to integrin cytoplasmic domains leads to recruitment of further proteins, forming a complex hierarchical structure that directs cytoskeletal assembly and signal transduction cascades [7].

It has been shown that talin and α-actinin are important in the assembly of focal adhesions [8]. Talin has actin-binding sites, and binding sites for the cytoskeletal protein vinculin. Because vinculin can also bind F-actin, it may cross-link talin and actin, thereby stabilizing the interaction. Vinculin stabilizes focal adhesions and transfers mechanical stress that drives cytoskeletal remodelling [9]. PtdIns(4,5)P_2 dissociates vinculin's head–tail interaction, unmasking its talin- and actin-binding sites [10].

Integrin-dependent activation of pp125FAK, which is also a component of focal adhesions, and subsequent tyrosine phosphorylation, also seem to be required for focal adhesion assembly. Inhibition of pp125FAK phosphorylation by tyrosine kinase inhibitors is associated with reduced formation of focal adhesions and stress fibres [11,12].

Cytoplasmic sequences in the α-subunit also appear to play a role in normal ligand-dependent localization of integrins to focal adhesions [13,14] and in cell motility [15,16]. A few proteins have been identified so far that interact with sequences of the α cytoplasmic domains, and might be involved in these processes [17,18].

Integrin $\alpha1\beta1$ is a receptor for collagens and laminins [19,20]. Its α-subunit is known to recruit the adapter protein, Shc, via the protein caveolin-1 [21]. The α1-subunit has a shorter cytoplasmic tail (15 amino acids) than any other α integrin subunit. It contains the Gly-Phe-Phe-Lys-Arg motif, which is conserved in all α integrin subunits, and the very basic α1-specific sequence Pro-Leu-Lys-Lys-Lys-Met-Glu-Lys.

In previous studies we showed that the cytoplasmic α1 tail is required for post-ligand events, such as actin stress-fibre formation and cell spreading [22]. In the present paper we report that the cytoplasmic domain of α1 is also required for the protein–protein interactions that accompany the formation of focal adhesions and tyrosine phosphorylation of focal adhesion components. Its involvement in this process is confirmed by the fact that this sequence serves as a binding site for the focal adhesion components paxillin, talin, α-actinin and pp125FAK. Binding of these proteins seems to be required for integrin function, since when peptides that mimic the cytoplasmic domain of α1 are transferred into cells, they compete for cytoplasmic α1 binding partners and influence $\alpha1\beta1$-specific adhesion. For α1 integrin–protein binding, the Lys-Ile-Gly-Phe-Phe-Lys-Arg motif

Abbreviations used: pp125FAK, focal adhesion kinase; CHO, Chinese hamster ovary.

[1] To whom correspondence should be addressed (e-mail kdanker@zedat.fu-berlin.de).

and, in particular, its two lysine residues, are important. In cells expressing an $\alpha1\beta1$ integrin which lacks the $\alpha1$ cytoplasmic domain, no obvious focal adhesions can be visualized by immunofluorescence studies.

MATERIALS AND METHODS

Cells, antibodies and matrix molecules

Chinese hamster ovary (CHO)-$\alpha1$/f.l., CHO-$\alpha1$/GFFKR and CHO-$\alpha1$/K cells, described previously [22], were stably transfected with the full-length cDNA of the rat $\alpha1$ integrin subunit and two cytoplasmically truncated $\alpha1$ cDNA constructs. One deletion construct yields a protein with eight amino acids deleted from the $\alpha1$ cytoplasmic domain ($\alpha1$/GFFKR), whereas the other lacks the entire cytoplasmic domain, except for the membrane-proximal lysine ($\alpha1$/K). All cell lines express collagen/laminin-binding $\alpha1\beta1$ integrin heterodimers on the cell surface. Stable CHO cell lines were cultured in α-modified Eagle's medium supplemented with 10 % (v/v) inactivated fetal calf serum (Eurobio, Raunheim, Germany), 50 units/ml penicillin/streptomycin, 440 μg/ml glutamine and 0.5 g/l G418 (Gibco BRL). PC12 cells were cultured in RPMI 1640 supplemented with 10 % (v/v) inactivated horse serum, 50 units/ml penicillin/streptomycin and 440 μg/ml glutamine.

For Western-blot analyses, rabbit polyclonal antibodies raised against rat $\alpha1$ (As2K5) and $\beta1$ (As3K4) integrin subunits were chosen. The antibodies have been described previously [23,24]. Monoclonal antibodies to pp125FAK (clone 77) and paxillin (clone 349) were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). The monoclonal antibodies to phosphotyrosine residues (clone PT66), vinculin (clone VIN-11-5), talin (clone 8d4) and the polyclonal anti-α-actinin, and all secondary antibodies were purchased from Sigma. Laminin and collagen were also purchased from Sigma.

Peptides and preparation of affinity matrices

Synthetic peptides used in this study were synthesized according to standard methods. They were purified and analysed by reverse-phase HPLC and matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry. Each peptide was supplemented by an additional N-terminal cysteine residue for immobilization to the carrier (activated thiol–Sepharose; Pharmacia) according to the manufacturer's instructions. Coupling efficiency was estimated by monitoring remaining peptides in solution by use of trinitrobenzoic acid [25]. Usually, 2 mmol peptide were coupled per ml of Sepharose. Any unreacted residual groups of activated thiol–Sepharose were inactivated with 2-mercaptoethanol. The affinity matrices were washed and stored in Tris-buffered saline containing 0.02 % (w/v) NaN_3. Cysteine-blocked Sepharose, used for control precipitations, was prepared similarly by adding 10 mmol cysteine in Tris-buffered saline/ml activated thiol–Sepharose. Before use, beads were blocked with 1 % (w/v) BSA in Tris-buffered saline.

Protein binding studies and Western-blot analysis

Affinity precipitation was performed according to the method of Schaller et al. [3]. Briefly, PC12 cells were lysed in 1 % (v/v) Triton X-100, 50 mM Tris/acetate, 50 mM NaCl, pH 7.6, 10 mM EGTA and 2 mM $MgCl_2$, containing 125 μg/ml aprotinin and leupeptin. The extract was centrifuged at 100000 ***g*** for 20 min at 4 °C. The protein concentration was adjusted to 0.5 mg/ml, and 500 μl of the extract were incubated with 20 μl of peptide–Sepharose for at least 1 h at 4 °C. The beads were pelleted, washed and eluted with SDS/PAGE sample buffer. Eluates were analysed by reducing SDS/PAGE, with subsequent immunoblotting with the aforementioned antibodies. Blots were developed by chemiluminescence.

Immunoprecipitation analysis

Immunoprecipitations were carried out as described earlier [22]. In brief, cell lysates were preincubated with 100 μl of 10 % (w/v) Protein G–Sepharose to remove unspecific binding. Protein G–Sepharose was pelleted and the supernatants were incubated with 10 μl of monoclonal anti-FAK antibody for 60 min at 4 °C. Immunocomplexes were recovered with 100 μl of 10 % (w/v) Protein G–Sepharose and washed five times with immunoprecipitation buffer (0.5 M NaCl, 50 mM Tris/HCl, pH 7.6, 2 mM $CaCl_2$, 0.05 % Nonidet P40, 1 mg/ml ovalbumin, 1 mM PMSF, and aprotinin, leupeptin, pepstatin A, each at 10 μg/ml). Bound proteins were eluted in 50 μl of sample buffer [50 mM Tris/HCl, pH 6.8, 0.025 % Bromophenol Blue, 10 % SDS, 20 % (v/v) glycerol and 25 % mercaptopropandiol] by boiling. Precipitates were further analysed by Western blots with the monoclonal phosphotyrosine-specific antibody PT66.

Cell loading with peptides and adhesion assays

PC12 cells and CHO-$\alpha1$/f.l. transfectants were suspended to a concentration of 5×10^6 cells/ml in serum-free medium and placed on ice for 10 min. Synthetic peptides were added to a final concentration of 1 mM and cells were immediately electroporated at 280 V, 960 μF (GenePulser; Bio-Rad Laboratories, München, Germany) using a 5 mm cuvette. After electroporation cells were kept on ice for a further 10 min. Viability of electroporated cells was monitored by Trypan Blue exclusion and counting. The matrix adhesion of PC12 and CHO-$\alpha1$/f.l. cells was carried out essentially as described in [23]. Microplates (96-well) were coated with collagen IV or laminin (Sigma) at 4 °C overnight at a concentration of 20 μg/ml in PBS, and blocked with 1 % (w/v) BSA in PBS for 30 min at 37 °C. A total of 5×10^4 electroporated cells was plated into each microtitre well. After 90 min incubation at 37 °C, non-attached cells were rinsed away with PBS and attached cells were fixed with 1 % glutardialdehyde in doubly distilled water, before staining with Crystal Violet [26]. Following dye solubilization, absorbance at 570 nm was measured using a microplate reader.

Immunofluorescence analysis

Glass coverslips were coated with 20 μg/ml collagen IV in PBS at 4 °C overnight. Coverslips were washed with PBS and blocked for 1 h at 37 °C with 1 % BSA. Cells were kept in suspension for 15 min, plated on to coverslips in α-modified Eagle's medium under serum-free conditions and allowed to adhere for 1 h. Cells were fixed with 3 % paraformaldehyde for 15 min, permeabilized with 0.1 % Triton X-100 in PBS for 2 min and rinsed twice. Blocking was performed with 1 % BSA for 20 min. After washing the coverslips with PBS, proteins were incubated with antibodies to paxillin, vinculin or PT66 for 2 h and washed four times with PBS. Cells were then visualized with FITC-labelled goat anti-mouse IgG and photographed with a Zeiss Axiolab microscope at 100× magnification.

RESULTS

Participation of $\alpha1$ integrin cytoplasmic domain in the formation of focal adhesions

The interactions of cytoplasmic domains of integrins with the cytoskeleton in focal adhesions involve the association of a

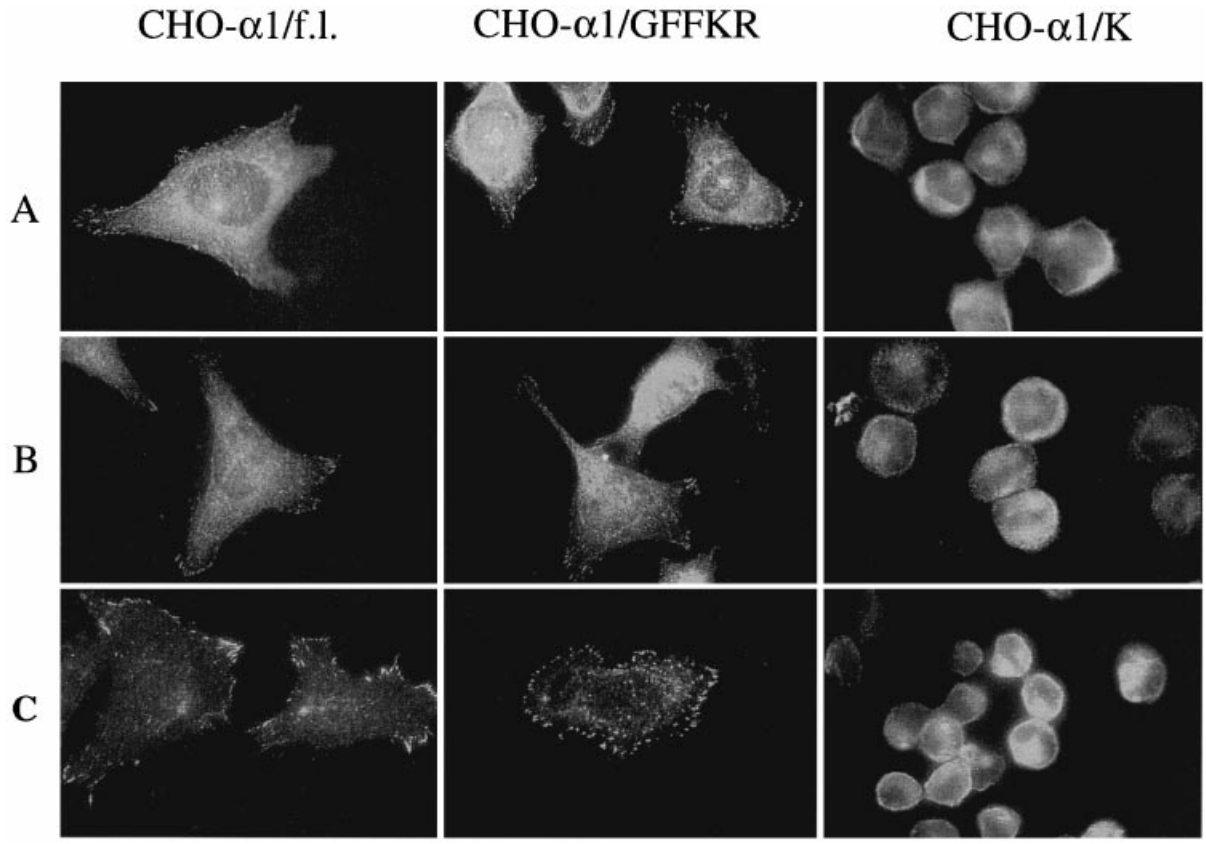

Figure 1 Immunofluorescence analyses of focal adhesions in CHO-α1/f.l., CHO-α1/GFFKR and CHO-α1/K cells

Cells expressing equal amounts of the respective α1 integrin on the cell surface were plated for 1 h on collagen IV, fixed, permeabilized and stained with antibodies against paxillin (**A**), vinculin (**B**) and phosphotyrosine residues (**C**), and subsequently incubated with FITC-labelled secondary goat anti-mouse antibody. CHO-α1/f.l. cells revealed normal focal adhesions that contain paxillin and vinculin, and localized tyrosine phosphorylation. CHO-α1/GFFKR also formed focal adhesions which contain paxillin, vinculin and tyrosine phosphorylation, but CHO-α1/K cells adhering to collagen IV are not able to spread and to form focal adhesions. Magn. ×100.

number of proteins. We addressed the question of whether parts of the cytoplasmic domain of the α1 integrin subunit participate in focal adhesion formation. cDNA coding for the whole rat α1 (α1/f.l.) integrin subunit and two constructs that give rise to α1 subunits with truncated cytoplasmic domains (α1/GFFKR, α1/K) were generated as described previously [22]. CHO cells that had been transfected with those constructs were plated for 1 h on collagen IV, fixed, permeabilized and stained with antibodies against paxillin, vinculin and phosphotyrosine residues (Figure 1A, 1B and 1C respectively). Both CHO-α1/f.l. and CHO-α_1/GFFKR cells formed focal adhesions containing paxillin, vinculin and tyrosine phosphorylation. In CHO-α1/K cells, which adhere to collagen IV but retain a spherical appearance on this α1β1-specific substrate, a similar staining pattern of these proteins was not observed. On fibronectin, focal adhesion formation of CHO-α1/K cells is not disturbed (results not shown).

Since the phosphotyrosine staining of focal adhesions is abolished in CHO-α1/K cells, we examined whether this phenomenon is reflected in proteins such as pp125FAK and paxillin which are phosphorylated on tyrosine residues upon matrix adhesion. For this reason we performed immunoprecipitation assays of pp125FAK and paxillin from CHO-α1/f.l., CHO-α1/GFFKR and CHO-α1/K cells kept in suspension or plated on poly-L-lysine, collagen IV or fibronectin. Subsequently, the phosphotyrosine signal was analysed by a phosphotyrosine-specific antibody (Figure 2). While CHO-α1/f.l. and CHO-α1/GFFKR cells that adhere to fibronectin and collagen IV reveal an increased tyrosine phosphorylation compared with

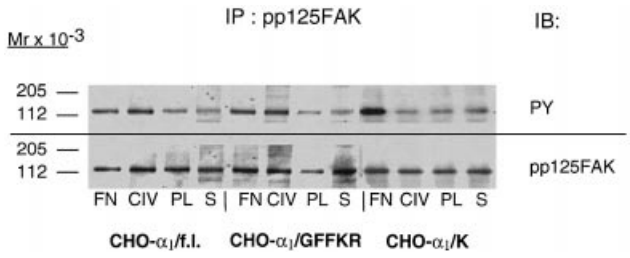

Figure 2 Tyrosine phosphorylation of pp125FAK of CHO-α1/f.l., CHO-α1/GFFKR and CHO-α_1/K cells

Cells were plated on collagen IV (CIV), fibronectin (FN) or poly-L-lysine (PL), or kept in suspension (S) for 1 h. After lysis, pp125FAK was immunoprecipitated. Subsequently, tyrosine phosphorylation of the precipitated protein was analysed in Western-blot analysis with a phosphotyrosine-specific antibody (PT66; upper panel). Half of each immunoprecipitate was further analysed with a monoclonal anti-FAK antibody to verify that each sample contains the same amount of pp125FAK (lower panel).

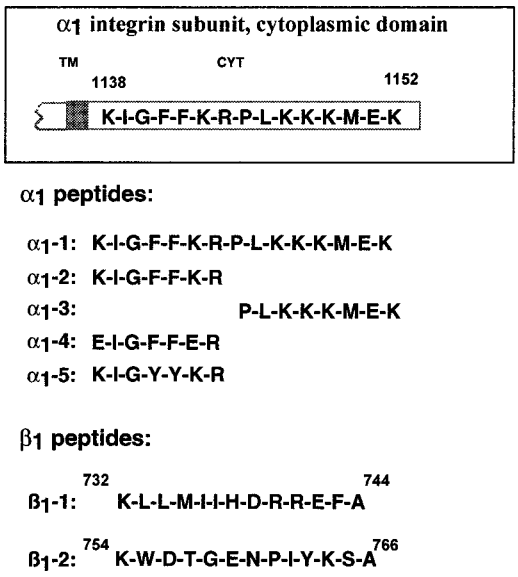

Figure 3 Sequences of peptides used in *in vitro* binding experiments

α1-1, complete cytoplasmic tail of the α1 integrin subunit (homologue in α1 subunits of variant species); α1-2, common regulatory sequence found in all cytoplasmic domains of α-subunits; α1-3, basic C-terminal portion of α1 subunit; α1-4 and α1-5, modified sequences of α1-2; β1-1 and β1-2, pp125FAK, α-actinin and paxillin binding (β1-1) or non-binding (β1-2) sequences were derived from the cytoplasmic domain of the β1 integrin chain as described in [3] and [33]; β1-1 is identical with SP1, β1-2 is identical with SP3. To enable orientated peptide immobilization to thiol-activated Sepharose, peptides were synthesized with an additional cysteine residue located at the N-terminus (results not shown).

cells which are kept in suspension or plated on poly-L-lysine, in CHO-α_1/K cells tyrosine phosphorylation of pp125FAK is not increased if cells adhere to collagen IV. This also observed for tyrosine phosphorylation of paxillin (results not shown).

In vitro binding assays with various cytoplasmic α1- and β1-specific peptides

Since focal adhesions are not properly formed in cells expressing the α1/Kβ1 integrin, and tyrosine phosphorylation of pp125FAK and paxillin also depends on the cytoplasmic domain of the α1 integrin subunit, we investigated whether focal adhesion proteins are capable of interacting with the cytoplasmic tail of the α1 integrin subunit. For this purpose we performed *in vitro* binding assays to determine whether cytoplasmic proteins bind to α1-specific peptides displaying the complete cytoplasmic tail (α1-1), or to the shorter membrane proximal sequence Lys-Ile-Gly-Phe-Phe-Lys-Arg (α1-2; Figure 3) immobilized to thiol-activated Sepharose. This approach has been successfully used to identify proteins that bind to sequences of the β1 cytoplasmic tail [2,3]. Lysates of PC12 cells, which express endogenous α1β1 integrin, were used as a source of cytoplasmic proteins. Western-blot analysis of proteins retained by the peptide Sepharoses revealed that paxillin, α-actinin and talin, as well as pp125FAK, bound to the full-length α1 cytoplasmic peptide (α1-1; Figure 4A). Vinculin did not bind to this peptide.

Furthermore, neither α1 nor β1 integrin subunits bound to the full-length α1 cytoplasmic peptide, indicating that detected interactions with the α1-1 peptide were not due to complexed proteins pulled down by interacting α1 or β1 subunits (Figure 4A). Protein–α1-1 interactions were also observed with the

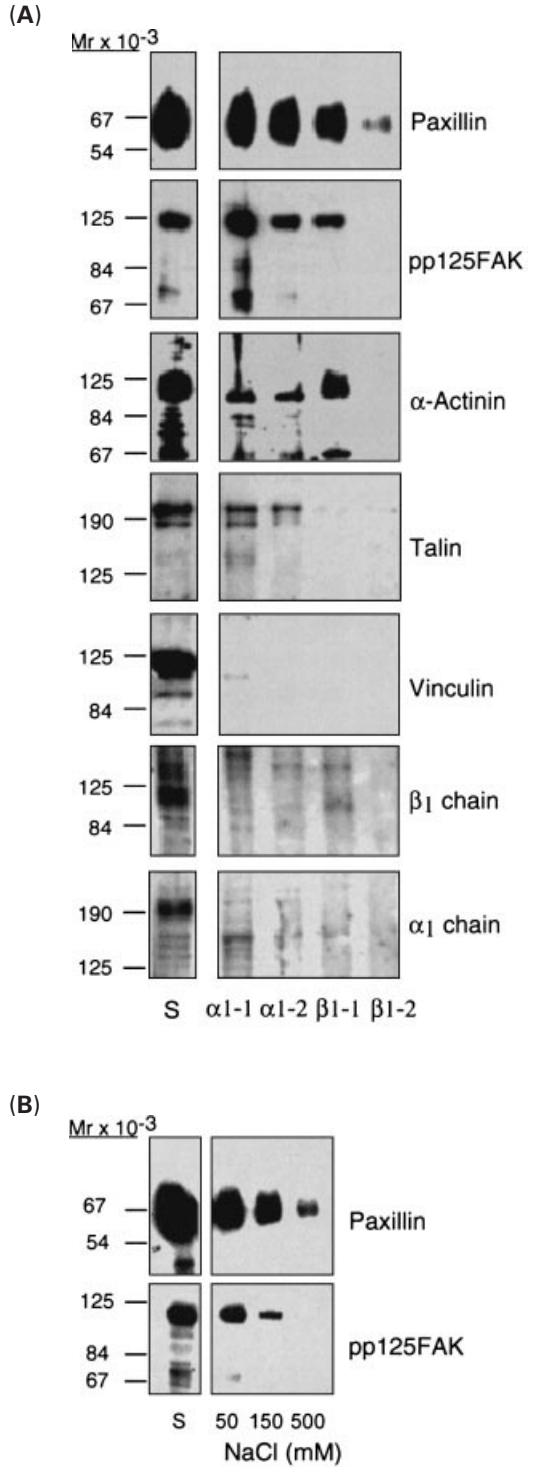

Figure 4 Binding of intracellular proteins from PC12 cell lysates to synthetic peptides displaying sequences of the cytoplasmic tails of α1 and β1 integrin subunits

Protein binding was examined as described in the Materials and methods section. The nomenclature and sequences of selected peptides are shown in Figure 3. β1A-specific peptides were used as positive (β1-1) and negative (β1-2) controls for cytoplasmic protein binding, as described earlier [3,33]. Peptide–Sepharose-complexed proteins were eluted from beads, separated by reducing SDS/PAGE and detected by immunoblotting with the appropriate antibodies (**A**). (**B**) The relation between protein binding efficiency and ion strength. PC12 cell lysates were adjusted to the indicated NaCl concentrations prior to incubation with α1-1 peptide Sepharoses. Abbreviation: S, PC12 cell lysate.

peptide α1-2 containing the conserved Gly-Phe-Phe-Lys-Arg motif.

To characterize the nature of the identified peptide–protein interactions we tested the binding of paxillin and pp125FAK to the α1-1 peptide in different NaCl concentrations. PC12 cell lysates were adjusted to different NaCl concentrations prior to incubation with peptide Sepharose. Figure 4(B) shows that paxillin and pp125FAK binding were decreased at 150 mM, and were almost zero at 500 mM NaCl.

To confirm the specificity of protein binding, peptides displaying sequences of the cytoplasmic tail of the β1A subunit were used as control targets. The β1-1 sequence, which is known to display a specific interaction site for cytoplasmic proteins [3], binds paxillin, pp125FAK and α-actinin. Talin does not bind to this sequence. The β1-2 peptide, which has been published as a non-interacting sequence, showed no protein binding (Figure 4A). Non-specific protein binding by this assay was further excluded by performing the assay with Sepharose derivatized by cysteine or a non-integrin peptide (NLTEHKPSTSSHNLG). However, both Sepharoses showed no affinity with any of the tested proteins (results not shown).

Examination of sequence specificity of protein binding to the α1 cytoplasmic tail

The sequence Gly-Phe-Phe-Lys-Arg is a conserved motif shared by all α-integrin subunits. In order to elucidate which amino acids of the Gly-Phe-Phe-Lys-Arg sequence are responsible for protein binding, we tested further peptides (see Figure 3) for cytoplasmic protein binding (Figure 5). Binding of talin, paxillin and pp125FAK to the Gly-Phe-Phe-Lys-Arg motif is disturbed if the two basic lysine residues are substituted by acidic glutamate residues (α1-4; Figure 5). Substitution of the two apolar phenylalanines by polar tyrosine (α1-5) had no influence on protein binding. Vinculin, which did not bind to the full-length α1 cytoplasmic sequence or to β1 sequences, also failed to bind these other peptides.

Beyond the Gly-Phe-Phe-Lys-Arg motif, α-integrin subunits are composed of a sequence that is specific for the particular α-subunit. Since the α1-subunit is characterized by its highly positively charged Pro-Leu-Lys-Lys-Lys-Met-Glu-Lys C-terminus, we addressed the question of whether this motif is further involved in protein binding. Whereas talin binding seems to be restricted to the Gly-Phe-Phe-Lys-Arg motif (α1-2; Figure 5), paxillin and pp125FAK also bound, to a lesser extent, with the α1-specific C-terminal sequence (α1-3).

Influence of the α1 cytoplasmic domain on the adhesion of CHO-α1/f.l. and PC12 cells

The functionality of the α1-peptide–protein binding observed in *in vitro* binding experiments was tested in cell matrix adhesion assays. PC12 cells and CHO-α1/f.l. cells were loaded with an α1-specific peptide, which should then compete for intracellular binding partners during adhesion to $\alpha1\beta$1-specific substrates. Cells that were electroporated without peptide were set as 100 %. Figure 6 shows that transfer of the α1-1 peptide reduced adhesion to collagen IV to 25 % in PC12 cells (Figure 6A) and to 20 % in CHO-α1/f.l. cells (Figure 6B). On laminin, the α1-1 peptide reduces adhesion of CHO-α1/f.l. cells to 25 % (Figure 6D), but is less effective in PC12 cells (Figure 6C). Loading with the α1-2 peptide, consisting only of the Gly-Phe-Phe-Lys-Arg motif, leads to a reduction of collagen IV adhesion (10 %) in PC12 cells, as well as in CHO-α1/f.l. cells (20 %). Adhesion to laminin of both cell lines is also decreased significantly by this peptide. Substitution of lysines with glutamates in the α1-4 peptide, which showed no protein binding, had no influence on adhesion to collagen IV, whereas peptide α1-5, which allows binding of talin, paxillin and pp125FAK, reduces adhesion to collagen IV to 60 % in both cell lines. The α1-specific membrane distal sequence Pro-Leu-Lys-Lys-Lys-Met-Glu-Lys (α1-3) had no effect on adhesion. The β1-1 peptide from the cytoplasmic domain of the β1 integrin chain caused a substantial decrease in the adhesion to collagen IV and laminin in both cell lines. β1-2, which did not bind any of the examined proteins, had no effect on matrix adhesion.

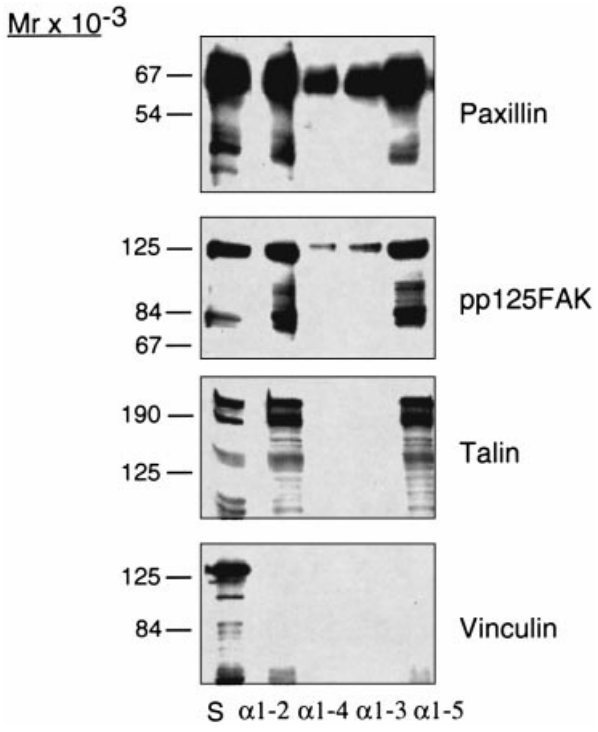

Figure 5 Intracellular protein binding to synthetic peptides derived from the cytoplasmic domain of the α1 integrin subunit

The interaction of the cytoplasmic proteins of PC12 cell lysates with different peptide Sepharoses was investigated (see Figure 2). Notice that (i) the change of the polarity within the sequence of peptide α1-2 (substitution of two lysine residues by two glutamate residues, peptide α1-4) reduces protein binding; (ii) the substitution of the two phenylalanines by tyrosine (peptide α1-5) has no influence on protein binding; and (iii) the basic C-terminal residue of the cytoplasmic α1 tail (peptide α1-3) does not display significant affinity for talin, but that paxillin, and to a lesser extent, pp125FAK binds to this sequence. Abbreviation: S, PC12 cell lysate.

DISCUSSION

Evidence is shown in the present paper that the very short (15 amino acids) α1 cytoplasmic tail of $\alpha1\beta$1 integrin is involved in focal adhesion formation and tyrosine phosphorylation of focal adhesion components. Immunofluorescence analysis of cells expressing an $\alpha1\beta$1 integrin, but lacking the α integrin-specific Gly-Phe-Phe-Lys-Arg motif, has shown that these cells are unable to form proper focal adhesions or actin stress fibres. Involvement of the α1 cytoplasmic domain in focal adhesion formation was confirmed by *in vitro* binding assays, which in particular revealed that the conserved Gly-Phe-Phe-Lys-Arg sequence acts as a target for cytoplasmic proteins such as α-actinin, talin, paxillin and pp125FAK. Uncoupling of these interactions by transferring surplus Gly-Phe-Phe-Lys-Arg-containing peptides into $\alpha1\beta$1-expressing cells led to a reduction of $\alpha1\beta$1-specific adhesion.

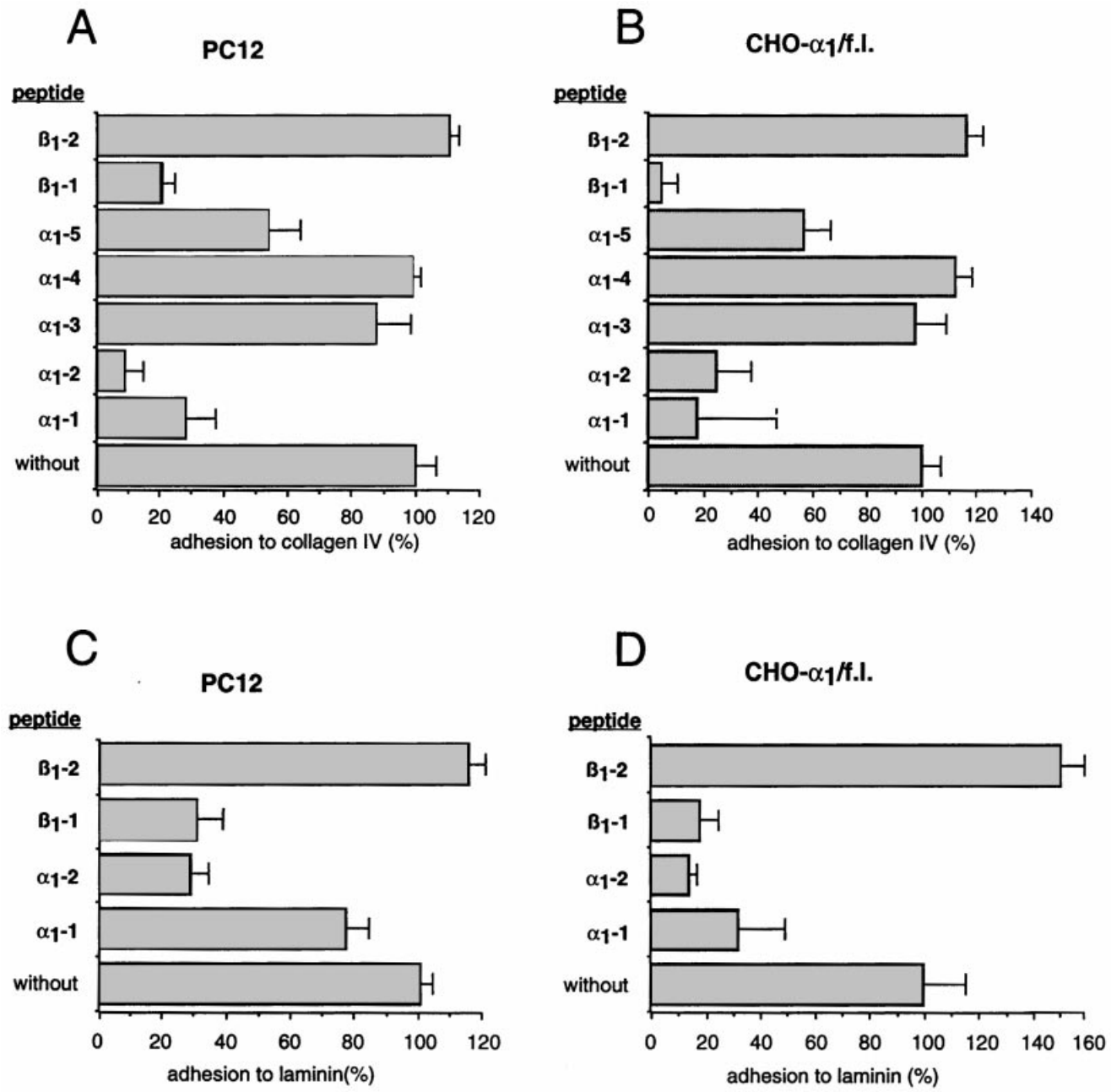

Figure 6 Adhesion of PC12 cells and CHO-α1/f.l. cells to collagen IV and laminin following intracellular peptide loading

PC12 cells (**A**, **C**) and CHO-α1/f.l. cells (**B**, **D**) were loaded with synthetic peptides by electroporation, as described in the Materials and methods section. Viability of cells after electroporation was assessed by Trypan Blue staining. Following electroporation, cells were plated on to 96-microwell plates coated with collagen IV (**A**, **B**) or laminin (**C**, **D**; 1×10^5 cells/well) and allowed to attach within 90 min. Following removal of non-attached cells, adhering cells were quantified by staining with Crystal Violet. Data are expressed as a percentage of the adhesion displayed by non-peptide-treated electroporated cells and are mean values of at least four independent experiments done in triplicate.

Within the membrane proximal Lys-Ile-Gly-Phe-Phe-Lys-Arg sequence, both lysines seem to be important for protein binding and, therefore, for integrin function.

The initial step in integrin-mediated signalling is the formation of focal adhesion complexes [7,27]. Numerous publications [14,28–30] have shown that the cytoplasmic tail of the β-subunit is important for this process. The involvement of the cytoplasmic domains of α-integrin subunits in post-ligand events varies and depends on the particular integrin [15,31]. Deletion of the cytoplasmic domain of the α5-integrin subunit leads to, at least partial, inhibition of stress fibre formation [16]. On $\alpha 1\beta 1$-specific substrates, stress fibre formation is completely inhibited in CHO cells carrying an $\alpha 1\beta 1$ integrin lacking the cytoplasmic tail of the α1 subunit [22]. In the present study we demonstrated that the α1 integrin cytoplasmic domain is also required for proper focal adhesion formation. Immunofluorescence analyses of CHO-α1/f.l. cells, using antibodies against focal adhesion components, revealed well-stained focal adhesions. In contrast, CHO-α1/K

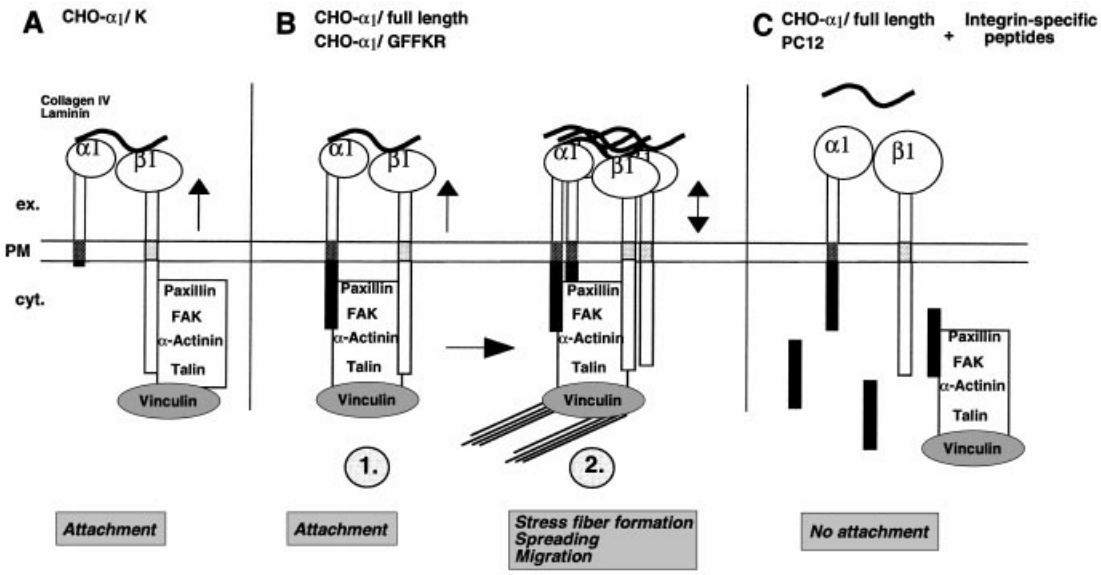

Figure 7 Possible involvement of the cytoplasmic domain of the α1 integrin subunit in $\alpha1\beta1$-mediated post-ligand events

For focal adhesion formation (1) and subsequent spreading (2), binding of focal adhesion components, not only to the cytoplasmic domain of the β1 integrin subunit, but also to the α1 subunit (in particular the Gly-Phe-Phe-Lys-Arg motif), seems to be required. This situation is reflected in CHO-α1/f.l. and in CHO-α1/GFFKR cells (**B**). If protein binding to the cytoplasmic domain of the α1-subunit is disturbed, as it is the case in CHO-α1/K cells, cells are still able to attach, but post-attachment events like spreading and migration are inhibited (**A**). α1-Specific peptides compete for the α1–protein interactions and the β1–protein interactions since the binding partners are at least partially the same. If the β1–protein interactions are inhibited cells are not able to attach (**C**). This has been shown for CHO-α1/f.l. and PC12 cells. Black lines, actin stress fibres; black bars, peptides, representing cytoplasmic integrin sequences; ex., extracellular; PM, plasma membrane; cyt., cytosol; arrow, integrin is able to bind ligand; double arrow, integrin is able to bind ligand (1) and post-ligand events are induced (2).

cells lack these structures. In CHO cells, which express an α1 subunit still containing the Gly-Phe-Phe-Lys-Arg motif, paxillin, vinculin and tyrosine phosphorylation are accumulated in focal adhesion structures.

The importance of the α1 cytoplasmic domain, and in particular its Gly-Phe-Phe-Lys-Arg motif, for focal adhesion formation was confirmed in *in vitro* binding assays. It was found that the focal adhesion proteins paxillin, pp125FAK, α-actinin and talin bind to a peptide representing the entire cytoplasmic domain of α1. Vinculin, which is known not to bind directly to integrins, could not be detected with α1-specific or β1-specific peptides. Recently [18], it has been reported that paxillin binds to the cytoplasmic domain of the α4-integrin subunit. This association is functional and markedly enhances the rates of integrin-dependent phosphorylation of pp125FAK and migration. Talin binds to a lesser extent to this cytoplasmic domain. Binding of α-actinin and vinculin was not observed in this study [18]. The target sequence for protein binding of the α1 integrin cytoplasmic domain seems to include the conserved Lys-Ile-Gly-Phe-Phe-Lys-Arg motif, because pp125FAK and talin also bind to this shortened peptide. The importance of this sequence as a target for protein binding is confirmed by *in vitro* binding experiments, in which the two lysine residues of the Lys-Ile-Gly-Phe-Phe-Lys-Arg sequence are replaced by acidic glutamate residues. Binding of pp125FAK and talin to this motif is markedly reduced. In previous studies it has been shown that the Gly-Phe-Phe-Lys-Arg sequence is a target binding sequence for many of the cytoplasmic α-subunit-binding proteins identified so far [17,32].

All identified α1-binding proteins have also been shown to interact with sequences of the cytoplasmic domain of the β1-subunit [1,3,33]. While paxillin, α-actinin and pp125FAK interact with the β1-1 peptide, talin binds further downstream of this sequence [30].

The importance of the Gly-Phe-Phe-Lys-Arg motif for integrin function is further confirmed by adhesion assays, in which PC12 and CHO-α1/f.l. cells were loaded with integrin-specific peptides before each assay. The use of integrin peptides mimicking sequences of the cytoplasmic domain of β-integrin subunits and acting as dominant negative inhibitors has been described before [34,35]. Transfer of Gly-Phe-Phe-Lys-Arg-containing peptides, but not of the α1-specific Pro-Leu-Lys-Lys-Lys-Met-Glu-Lys peptide, leads to significantly reduced adhesion to $\alpha1\beta1$-specific substrates in PC12 and CHO-α1/f.l. cells. Adhesion of PC12 cells to laminin is only slightly decreased by the α1-1 peptide. This effect might be due to the fact that PC12 cells express another collagen/laminin-binding integrin, $\alpha3\beta1$, that primarily supports laminin adhesion, but not adhesion to collagen IV [36]. The Gly-Phe-Phe-Lys-Arg peptide that is also part of the α3-integrin subunit blocks adhesion of PC12 cells to laminin to a greater extent. Adhesion of CHO-α1/f.l. cells and of PC12 cells is reduced to a similar extent by the functionally important β1-1 peptide.

Our findings point to an interaction of the α1-integrin subunit, in particular of the conserved Gly-Phe-Phe-Lys-Arg motif, with the components of the focal adhesions. Our recent model suggests that for focal adhesion formation and subsequent spreading, binding of focal adhesion components, not only to the cytoplasmic domain of the β1 integrin subunit, but also to the α1 subunit, is required. This situation is reflected in CHO-α1/f.l. and in CHO-α1/GFFKR cells (Figure 7B). If protein binding to the cytoplasmic domain of the α1-subunit is disturbed, as is the case in CHO-α1/K cells, cells are still able to attach, but post-attachment events like spreading and migration are inhibited

(Figure 7A). α1-Specific peptides compete for the α1–protein interactions and the β1–protein interactions, since the binding partners are at least partially the same. If the β_1–protein interactions are inhibited cells are not able to attach (Figure 7C).

The question remains as to how all these proteins identified so far are able to interact with a sequence of only five amino acids. One possibility is that the proteins bind as a complex, since we used the mild non-ionic detergent Triton X-100 for solubilization. Another explanation is that the binding of the different proteins to the cytoplasmic α1 domain is a very dynamic process during matrix adhesion, in which the various proteins bind in subsequent steps. Future studies will address this question in detail.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 366, Da 310/3-1), the Sonnenfeld-Stiftung and the Fonds der Chemischen Industrie. We thank Dr A. Scott for improving the English of the manuscript.

REFERENCES

1 Horwitz, A., Duggan, K., Buck, C., Beckerle, M. C. and Burridge, K. (1986) Interaction of plasma membrane fibronectin receptor with talin – a transmembrane linkage. Nature (London) **20**, 531–533

2 Otey, C. A., Pavalko, F. M. and Burridge, K. (1990) An interaction between alpha-actinin and the beta 1 integrin subunit *in vitro*. J. Cell Biol. **111**, 721–729

3 Schaller, M. D., Otey, C. A., Hildebrand, J. D. and Parsons, J. T. (1995) Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. J. Cell Biol. **130**, 1181–1187

4 Hayashi, Y., Haimovich, B., Reszka, A., Boettiger, D. and Horwitz, A. (1990) Expression and function of chicken integrin beta 1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. J. Cell Biol. **110**, 175–184

5 Marcantonio, E. E., Guan, J. L., Trevithick, J. E. and Hynes, R. O. (1990) Mapping of the functional determinants of the integrin beta 1 cytoplasmic domain by site-directed mutagenesis. Cell Regul. **1**, 597–604

6 Solowska, J., Guan, J. L., Marcantonio, E. E., Trevithick, J. E., Buck, C. A. and Hynes, R. O. (1989) Expression of normal and mutant avian integrin subunits in rodent cells. J. Cell Biol. **109**, 853–861

7 Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K. and Yamada, K. M. (1995) Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J. Cell Biol. **131**, 791–805

8 Critchley, D. R., Holt, M. R., Barry, S. T., Priddle, H., Hemmings, L. and Norman, J. (1999) Integrin-mediated cell adhesion: the cytoskeletal connection. Biochem. Soc. Symp. **65**, 79–99

9 Ezzell, R. M., Goldmann, W. H., Wang, N., Parasharama, N. and Ingber, D. E. (1997) Vinculin promotes cell spreading by mechanically coupling integrins to the cytoskeleton. Exp. Cell Res. **231**, 14–26

10 Gilmore, A. P. and Burridge, K. (1996) Regulation of vinculin binding to talin and actin by phosphatidylinositol-4-5-bisphosphate. Nature (London) **381**, 531–535

11 Burridge, K., Turner, C. E. and Romer, L. H. (1992) Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J. Cell Biol. **119**, 893–903

12 Lyman, S., Gilmore, A., Burridge, K., Gidwitz, S. and White II, G. C. (1997) Integrin-mediated activation of focal adhesion kinase is independent of focal adhesion formation or integrin activation. Studies with activated and inhibitory beta3 cytoplasmic domain mutants. J. Biol. Chem. **272**, 22538–22547

13 Briesewitz, R., Kern, A. and Marcantonio, E. E. (1993) Ligand-dependent and -independent integrin focal contact localization: the role of the alpha chain cytoplasmic domain. Mol. Biol. Cell **4**, 593–604

14 Ylanne, J., Chen, Y., O'Toole, T. E., Loftus, J. C., Takada, Y. and Ginsberg, M. H. (1993) Distinct functions of integrin alpha and beta subunit cytoplasmic domains in cell spreading and formation of focal adhesions. J. Cell Biol. **122**, 223–233

15 Chan, B. M., Kassner, P. D., Schiro, J. A., Byers, H. R., Kupper, T. S. and Hemler, M. E. (1992) Distinct cellular functions mediated by different VLA integrin alpha subunit cytoplasmic domains. Cell (Cambridge, Mass.) **68**, 1051–1069

16 Bauer, J. S., Varner, J., Schreiner, C., Kornberg, L., Nicholas, R. and Juliano, R. L. (1993) Functional role of the cytoplasmic domain of the integrin alpha 5 subunit. J. Cell Biol. **122**, 209–221

17 Coppolino, M., Leung-Hagesteijn, C., Dedhar, S. and Wilkins, J. (1995) Inducible interaction of integrin alpha 2 beta 1 with calreticulin. Dependence on the activation state of the integrin. J. Biol. Chem. **270**, 23132–23138

18 Liu, S., Thomas, S. M., Woodside, D. G., Rose, D. M., Kiosses, W. B., Pfaff, M. and Ginsberg, M. H. (1999) Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. Nature (London) **402**, 676–681

19 Hemler, M. E., Sanchez-Madrid, F., Flotte, T. J., Krensky, A. M., Burakoff, S. J., Bhan, A. K., Springer, T. A. and Strominger, J. L. (1984) Glycoproteins of 210,000 and 130,000 m.w. on activated T cells: cell distribution and antigenic relation to components on resting cells and T cell lines. J. Immunol. **132**, 3011–3018

20 Kuhn, K. and Eble, J. (1994) The structural bases of integrin-ligand interactions. Trends Cell Biol. **4**, 256–261

21 Wary, K. K., Mariotti, A., Zurzolo, C. and Giancotti, F. G. (1998) A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. Cell (Cambridge, Mass.) **94**, 625–634

22 Vossmeyer, D., Kauffmann, C., Löster, K., Lucka, L., Horstkorte, R., Reutter, W. and Danker, K. (2000) The cytoplasmic domain of the alpha1 integrin subunit influences stress fiber formation via the conserved GFFKR motif. Exp. Cell Res. **256**, 321–327

23 Löster, K., Voigt, S., Heidrich, C., Hofmann, W. and Reutter, W. (1994) Cell-collagen adhesion is inhibited by monoclonal antibody 33.4 against the rat alpha 1-integrin subunit. Exp. Cell Res. **212**, 155–160

24 Löster, K., Baum, O., Hofmann, W. and Reutter, W. (1995) Chemical cross-linking leads to two high molecular mass aggregates of rat alpha 1 beta 1 integrin differing in their conformation but not in their composition. FEBS Lett. **373**, 234–238

25 Habeeb, A. F. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. **14**, 328–336

26 Gillies, R. J., Didier, N. and Denton, M. (1986) Determination of cell number in monolayer cultures. Anal. Biochem. **159**, 109–113

27 Aplin, A. E. and Juliano, R. L. (1999) Integrin and cytoskeletal regulation of growth factor signaling to the MAP kinase pathway. J. Cell Sci. **112**, 695–706

28 Ylanne, J., Huuskonen, J., O'Toole, T. E., Ginsberg, M. H., Virtanen, I. and Gahmberg, C. G. (1995) Mutation of the cytoplasmic domain of the integrin beta 3 subunit. Differential effects on cell spreading, recruitment to adhesion plaques, endocytosis, and phagocytosis. J. Biol. Chem. **270**, 9550–9557

29 Lewis, J. M. and Schwartz, M. A. (1995) Mapping *in vivo* associations of cytoplasmic proteins with integrin beta 1 cytoplasmic domain mutants. Mol. Biol. Cell **6**, 151–160

30 Kaapa, A., Peter, K. and Ylanne, J. (1999) Effects of mutations in the cytoplasmic domain of integrin beta(1) to talin binding and cell spreading. Exp. Cell Res. **250**, 524–534

31 Kassner, P. D., Alon, R., Springer, T. A. and Hemler, M. E. (1995) Specialized functional properties of the integrin alpha 4 cytoplasmic domain. Mol. Biol. Cell, **6**, 661–674

32 Wixler, V., Laplantine, E., Geerts, D., Sonnenberg, A., Petersohn, D., Eckes, B., Paulsson, M. and Aumailley, M. (1999) Identification of novel interaction partners for the conserved membrane proximal region of alpha-integrin cytoplasmic domains. FEBS Lett. **445**, 351–355

33 Otey, C. A., Vasquez, G. B., Burridge, K. and Erickson, B. W. (1993) Mapping of the alpha-actinin binding site within the beta 1 integrin cytoplasmic domain. J. Biol. Chem. **268**, 21193–21197

34 Buttery, P. C., Mallawaarachchi, C. M., Milner, R., Doherty, P. and ffrench-Constant, C. (1999) Mapping regions of the beta1 integrin cytoplasmic domain involved in migration and survival in primary oligodendrocyte precursors using cell-permeable homeopeptides. Biochem. Biophys. Res. Commun. **259**, 121–127

35 Liu, K. Y., Timmons, S., Lin, Y. Z. and Hawiger, J. (1996) Identification of a functionally important sequence in the cytoplasmic tail of integrin beta 3 by using cell-permeable peptide analogs. Proc. Natl. Acad. Sci. U.S.A. **93**, 11819–11823

36 Tomaselli, K. J., Hall, D. E., Flier, L. A., Gehlsen, K. R., Turner, D. C., Carbonetto, S. and Reichhardt, L. F. (1990) A neuronal cell line (PC12) expresses two beta 1-class integrins-alpha 1 beta 1 and alpha 3 beta 1-that recognize different neurite outgrowth-promoting domains in laminin. Neuron **5**, 651–662

Received 19 September 2000/31 January 2001; accepted 1 March 2001