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Ligand-dependent and -independent Integrin Focal Contact Localization: The Role of the α Chain Cytoplasmic Domain

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Many integrin receptors localize to focal contact sites upon binding their ligand. However, unoccupied integrin receptors do not localize to focal contact sites. Because the integrin β_1 cytoplasmic domain appears to have a focal contact localization signal, there must be a mechanism by which this domain is kept inactive in the unoccupied state and becomes exposed or activated in the occupied receptor. We considered that this mechanism involves the α subunit cytoplasmic domain. To test this hypothesis, we have established two NIH 3T3 cell lines that express either the human α_1 wild-type subunit (HA1 cells) or the cytoplasmic domain deleted α_1 subunit (CYT cells). Both cell lines express similar levels of the human α_1 subunit, and there is no significant effect of the deletion on the dimerization and surface expression of the receptor. Furthermore, the deletion had no effect on the binding or adhesion via $\alpha_1\beta_1$ to its ligand collagen IV. However, when these two cell lines are plated on fibronectin (FN), which is a ligand for $\alpha_5\beta_1$ but not for $\alpha_1\beta_1$, there is a striking difference in the cellular localization of $\alpha_1\beta_1$. The HA1 cells show only α_5 in focal contacts, without α_1, demonstrating that all of the integrin localization is ligand dependent. In contrast, when the CYT cells are plated on FN, the mutant α_1 appears in focal contacts along with the α_5/β_1. Thus, there is both ligand-dependent (α_5/β_1) and ligand-independent (α_1/β_1) focal contact localization in these cells. The truncated α_1 also localized to focal contacts in a ligand-independent manner on vitronectin. We conclude that the mutant α_1 no longer requires ligand occupancy for focal contact localization. These data strongly suggest that the α cytoplasmic domain plays a role in the normal ligand-dependent integrin focal contact localization.

INTRODUCTION

Many biological processes, such as morphogenesis, metastasis, and inflammation, involve integrins, a family of heterodimeric receptors. Integrin receptors each consist of an α and a β subunit that associate to mediate interactions among cells and also between cells and the extracellular matrix (ECM). Fourteen α subunits and eight β subunits have been identified thus far, and a subset of α subunits and β subunits are known to dimerize with more than one respective partner, increasing the number of distinct receptors (Hemler, 1990; Springer, 1990; Hynes, 1992). One receptor may bind various ligands and there is redundancy in ligand specificity. Fibronectin (FN), for example, is a ligand for $\geq$10 integrin receptors.

Many integrin receptors that are involved in adhesion concentrate in specific regions within the plasma membrane after having bound their ECM substrates. These specialized domains of the plasma membrane are recognized in interference reflection microscopy as dark areas and are called focal contact sites (Burridge *et al.*, 1988). The actin microfilament network is anchored at focal contact sites, which also contain other components of the cytoskeleton, such as vinculin, α-actinin, and talin (Chen *et al.*, 1985; Damsky *et al.*, 1985; Dejana *et al.*, 1988; Singer *et al.*, 1988). The latter two proteins have been shown to interact with the cytoplasmic domain of the β_1 subunit in vitro, suggesting a direct interaction in vivo (Horwitz *et al.*, 1986; Tapley *et al.*, 1989; Otey *et al.*, 1990). Thus,

integrins can provide a mechanical linkage between the ECM and the cytoskeleton.

All integrin subunits contain a large extracellular domain, a single transmembrane segment, and a carboxyl terminal cytoplasmic domain of varying length. The β_1 cytoplasmic domain is highly conserved in both vertebrates and invertebrates (DeSimone and Hynes, 1988; Marcantonio and Hynes, 1988). Previous work has shown that this domain is required for integrin focal contact localization. There are three regions that seem to be crucial as identified by site-directed mutagenesis studies (Hayashi *et al.*, 1990; Solowska *et al.*, 1989; Marcantonio *et al.*, 1990; Reszka *et al.*, 1992). Furthermore, it has been shown recently that the β_1 cytoplasmic domain itself is able to direct chimeric proteins to focal contacts. LaFlamme *et al.* (1992) constructed a chimeric protein consisting of the transmembrane and extracellular domains of the human interleukin-2 receptor and the integrin β_1 cytoplasmic domain. If cells expressing this protein are plated on FN, the interleukin-β_1 chimera colocalizes with endogenous FN receptors ($\alpha_5\beta_1$) in focal contacts. Geiger *et al.* (1992) have recently shown similar results using the extracellular portion of N-cadherin fused to the integrin β_1 transmembrane and cytoplasmic domains. These findings suggest that the β_1 cytoplasmic domain is not only necessary but may be sufficient to direct integrins to focal contact sites. However, the behavior of integrin heterodimers in vivo is different than the chimera. A number of authors have shown that integrin receptors do not localize to focal contact sites unless ligand has bound (Dejana *et al.*, 1988; Singer *et al.*, 1988). In addition, LaFlamme *et al.* (1992) showed that addition of a soluble ligand was effective in promoting receptor movement to focal contact sites, even when this ligand is not incorporated into the matrix on which the cell is bound. One explanation for these results would be that occupation of the integrin by ligand leads to a change in the receptor by which the β cytoplasmic domain becomes "active" or "unmasked." Thus, we believe that there is an inhibitory conformation or component of the normal integrin receptor, which prevents the β_1 cytoplasmic domain from being sufficient to localize to focal contact sites in the absence of ligand.

We have been using the human integrin $\alpha_1\beta_1$, which is a receptor for laminin and collagen (Kramer and Marks, 1989; Turner *et al.*, 1989; Hall *et al.*, 1990) as a system for understanding integrin structure and function (Briesewitz *et al.*, 1993). To test the role of the cytoplasmic domain of the α_1 integrin subunit in focal contact localization, we have deleted this segment and investigated whether it exerts an inhibitory effect on the focal contact localization signal in the β_1 subunit when this integrin has not bound ligand.

MATERIALS AND METHODS

Site-directed Mutagenesis and Plasmid Construction

Restriction enzymes and polynucleotide kinase were purchased from New England Biolabs (Beverly, MA), T4 DNA ligase from Bethesda Research Laboratories (Bethesda, MD), and Taq polymerase from Perkin-Elmer (Norwalk, CT). Standard recombinant DNA methods were used. Oligonucleotides were purchased from Operon Technologies (Alameda, CA) and were used directly without further purification. Polymerase chain reaction (PCR) was used to delete the human α_1 cytoplasmic domain. An in-frame termination codon was placed at the position of amino acid residue 1145 of the human α_1 cDNA (Briesewitz *et al.*, 1993), deleting the eight amino acids that immediately follow the GFFKR sequence. The carboxyl termini of the wild-type and the mutant protein are shown below:

Wild-type: GFFKRPLKKKMEK*

Deleted: GFFKR*

After mutagenesis, the mutant DNA sequence was confirmed by dideoxy sequencing (Sequenase, United States Biochemical, Cleveland, OH). The fidelity of all regions amplified by PCR was also verified by sequencing. Both the wild-type and the mutant human α_1 cDNAs were subcloned into a modified version of pLEN (Marcantonio *et al.*, 1990).

DNA Transfections

NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Cells (5×10^5) plated the previous day were cotransfected with 20 μg of pLEN-α_1 plasmids and 2 μg pSV2neo (Southern and Berg, 1982) as a calcium phosphate precipitate as described previously (Solowska *et al.*, 1989). After 3 d, the cells were split into DMEM supplemented with 10% FCS and 0.5 mg/ml G418 (Geneticin, GIBCO, Grand Island, NY). After 10–14 d, G418 resistant clones were isolated and screened by immunoprecipitation.

Antibodies

Rabbit anti-β_1 cytoplasmic domain antibodies and rabbit anti-α_5 cytoplasmic domain antibodies were prepared as described (Marcantonio and Hynes, 1988; Hynes *et al.*, 1989). Monoclonal anti-human α_1 (TS2/7) was a gift of T. Springer (Harvard Medical School). Monoclonal anti-human α_1 1B3 was a gift of I. Bank (Chaim Sheba Medical Center, Tel-Hashomer, Israel). Monoclonal anti-mouse vinculin was purchased from Sigma (St. Louis, MO), and rabbit anti-talin antibodies were kind gifts from K. Burridge (University of North Carolina) and Mary Beckerle (University of Utah). Rabbit inhibitory anti-rat β_1 (Lenny) antibodies were provided by C. Buck (Wistar Institute) (Albelda *et al.*, 1989).

Radiolabeling and Immunoprecipitation

Cells were labeled with Na ^{125}I (New England Nuclear, Boston, MA) and lactoperoxidase as a monolayer as described (Hynes, 1973). Cells (10^7) and 1–2 mCi/ml were used per experiment. The cells were extracted with 0.5% NP-40 and immunoprecipitated as described (Marcantonio and Hynes, 1988). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). Separation gels were 7.0% acrylamide with a 3% stacking gel. Samples were prepared in sample buffer (5% SDS, 100 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 6.8, 10 mM EDTA, 10% glycerol and bromophenol blue) and boiled for 3 min.

Adhesive Proteins

Human collagen type IV from placenta and intact mouse collagen type IV from Engel Breth-Holm-Swarm-tumors were isolated as de-

scribed previously (Vandenberg *et al.*, 1991). Human FN was isolated from plasma by affinity chromatography on gelatin-Sepharose (Pharmacia, Piscataway, NJ) as described previously (Engvall and Ruoslahti, 1977). Human vitronectin (VN) was purchased from GIBCO-BRL.

Affinity Chromatography

The 120-kDa fragment of fibronectin (FNf)[1] was prepared and coupled to Sepharose as described (Hynes *et al.*, 1989). Collagen IV was dialyzed against 0.1 M NaAc, 0.5 M NaCl, pH 8.3, and coupled to CNBr-activated Sepharose at 1 mg/ml according to the manufacturers' instructions. 3T3 cell lines were surface labeled with ^{125}I as described above. Cell extracts were prepared using either 50 mM β-octyl-glucoside (BOG) in 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 0.5 mM $MnCl_2$ (TCM) or 50 mM BOG in 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $MnCl_2$ (TMM). After a 10-min incubation on ice, detergent insoluble material was removed by centrifugation at 10 000 × *g* for 20 min. The resulting supernatant was incubated batch-wise with 100 μl of packed beads (either FNf-Sepharose or collagen IV-Sepharose) for 30 min at 4°C with end-over-end mixing. The beads were recovered by sedimentation and washed four times with either 50 mM BOG in TMM or 50 mM BOG in TCM followed by elution with 1 ml of 50 mM BOG in TMM containing 10 mM EDTA. This eluate was concentrated by immunoprecipitation with anti-β_1 antibody followed by SDS-PAGE analysis.

Immunofluorescence

FN (1 μg), collagen IV (0.5 μg), or VN (1 μg) in 70 μl of phosphate-buffered saline (PBS) was air-dried onto cover slips. After drying, the proteins were rehydrated in 100 μl over 30 min followed by two washes with PBS. Cells were incubated in serum-free media (DMEM) and supplemented with 25 μg/ml cycloheximide 2 h before trypsinization. Afterward, the reaction was stopped with serum, and the cells were washed in PBS twice and plated onto glass cover slips coated with matrix proteins. The cells were placed in the tissue culture incubator for 3 h in serum-free media plus cycloheximide. In some experiments, lysophosphatidic acid (50 ng/ml) (Sigma) was added 30 min before fixation. The cells were fixed in 4% paraformaldehyde in PBS for 5 min and permeabilized with 0.2% NP-40 in PBS for 5 min afterward. The antibodies against talin, vinculin, and α_5 were diluted 1:100 in 10% normal goat serum in PBS. TS2/7 hybridoma supernatant was supplemented with normal goat serum (10% final) and then used directly. The cover slips were overlaid with 20 μl of primary antibody solution and incubated at 37°C for 30 min. After washing in PBS, the coverslips were incubated with 20 μl of secondary antibodies for 30 min at 37°C. The secondary antibodies were F(ab')$_2$ fragments of rhodamine-conjugated rabbit anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG (Cappel, Durham, NC), diluted 1:250 in PBS/10% normal goat serum. All antibody preparations were centrifuged at 13 000 rpm for 10 min before use.

For experiments using staurosporine or phorbol esters, upon plating the cells were incubated with 50 nM of either staurosporine or phorbol myristate acetate (Sigma).

Cell Adhesion Assays

Adhesion assays were done essentially as described by Aumailley *et al.* (1989). Adhesive proteins were diluted in 0.1 M acetic acid (collagen IV) or distilled water (FN) and allowed to dry in microtiter wells at room temperature. After washing with PBS, remaining protein binding sites were blocked with bovine serum albumin (1% in PBS). Cells were suspended in serum-free DMEM (4–5 × 10^5/ml) and incubated in the wells for 45 min at 37°C. Nonadherent cells were removed by washing with PBS, and bound cells were fixed with 70% ethanol and stained with crystal violet (0.1% in distilled water). Excess stain was removed with water. Bound stain was dissolved with Triton X-100 (0.2% in distilled water) and the optical density was read at 595 nm.

For inhibition assays, cells were preincubated for 10 min at room temperature with mouse anti-human α_1 mAb 1B3.1 diluted 1:200 in DMEM (Bank *et al.*, 1989), with rabbit anti-rat β_1 serum (Lenny) diluted 1:100 in DMEM, or with 5 mM EDTA in DMEM and then transferred to wells coated with FN (0.2 μg/well) or collagen IV (0.1 μg/well). After 30 min at 37°C, nonadherent cells were removed and bound cells fixed and detected as described above. All experiments were done in triplicate.

RESULTS

Expression of Wild-Type and Cytoplasmic Domain Deleted Human α_1 Subunits in Mouse NIH 3T3 Cells

Using site-directed mutagenesis, we have placed a termination codon in the open reading frame of the human α_1 cDNA at the position of amino acid residue 1145, just 5′ of the cytoplasmic domain. Thus, the eight amino acids that compose the cytoplasmic domain of the wild-type human α_1 subunit are deleted in the mutant. To study the localization and adhesive properties of this mutation, we have cotransfected into mouse NIH 3T3 cells the mutant human α_1 cDNA in an expression vector along with a plasmid conferring neomycin resistance and selected positive clones. One NIH 3T3 cell line that expresses the cytoplasmic domain deleted human α_1 cDNA was studied further and designated CYT. The integrin profile of these cells was then compared with that of an NIH 3T3 line expressing the wild-type human α_1 subunit (HA1) (Briesewitz *et al.*, 1993). Figure 1 shows the results of an immunoprecipitation of extracts derived from ^{125}I labeled HA1 cells (Figure 1, a–c) and labeled

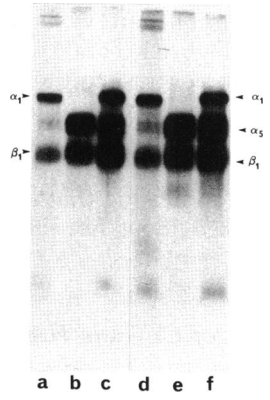


Figure 1. Expression of wild-type and cytoplasmic domain deleted human α_1 subunits in mouse NIH 3T3 cells. Extracts of ^{125}I surface-labeled clone HA1 cells (left) and CYT cells (right) were incubated with mouse monoclonal anti-human α_1 antibody TS2/7 (a and d), rabbit anti-α_5 peptide serum (b and e), or rabbit anti-β_1 cytoplasmic peptide serum (c and f). Immunoprecipitates were recovered by the use of protein A-Sepharose (b, c, e, and f) or by using goat anti-mouse agarose (a and d), followed by analysis using SDS-PAGE and autoradiography. Similar levels of surface α_1 expression and α/β heterodimer formation are seen for both the wild-type and mutant cell lines.

[1] Abbreviations used: FNf, 120-kDa fragment of fibronectin; PKC, protein kinase C; TCM, 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 0.5 mM $MnCl_2$; TMN, 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $MnCl_2$.

CYT cells (Figure 1, d–f). Immunoprecipitation of both extracts with an anti-β_1 antibody shows β_1 in complex with α subunits typical of NIH 3T3 cells (Solowska *et al.*, 1989) as well as an additional band of ~180-kDa, representing the exogenous human α_1 subunit coprecipitating with the endogenous β_1 subunit (Figure 1, c and f). The presence of authentic human α_1 was demonstrated by immunoprecipitation with a mouse monoclonal anti-human α_1 antibody (Figure 1a and d) that resulted in the precipitation of $\alpha_1\beta_1$ complexes from both cell lines, indicating that the exogenous human α subunit is assembled into integrin heterodimeric complexes. The relative amounts of the major FN receptor, $\alpha_5\beta_1$, in these cells is similar between the two lines (Figure 1, b and e). Quantitative determination of the relative counts in each of the major bands (α_1, α_5, and β_1) shows very similar amounts of these three subunits between the two 3T3 lines (corroborative data). Thus, the deletion of the α_1 cytoplasmic domain does not appear to affect dimerization with the endogenous mouse β_1 subunit, and the relative surface expression of the major integrins of the two 3T3 lines are similar, which allowed us to compare their relative adhesive and localization properties.

Next, we compared the binding properties of the mutant and wild-type integrins expressed on these two cells lines by affinity chromatography. ^{125}I-labeled extracts of HA1 and CYT cells were prepared as described in MATERIALS AND METHODS. These extracts were incubated with FNf-Sepharose or collagen IV-Sepharose, washed, and eluted with EDTA. The results of these experiments are shown in Figure 2. The affinity binding experiments were performed under two different conditions, either with Ca^{2+} (Figure 2, a–c and g–i) or with Mg^{2+} (Figure 2, d–f and j–l). Both the wild-type and mutant $\alpha_1\beta_1$ bound to collagen IV-Sepharose in a Mg^{2+}-dependent manner (Figure 2, f and l). They did not bind to FNf-Sepharose, in contrast to the FN receptor $\alpha_5\beta_1$ (Figure 2, e and k) that can bind its substrate in the presence of Ca^{2+} or Mg^{2+} (Figure 2, b, e, h, and k). Thus, the mutant and the wild-type $\alpha_1\beta_1$ showed similar binding specificities, indicating that deletion of the cytoplasmic domain of α_1 does not lead to a change in ligand binding.

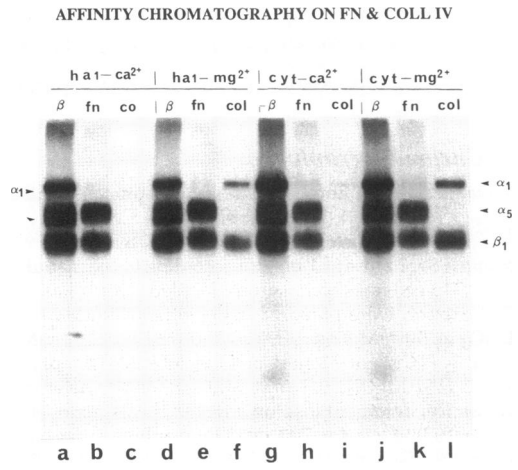


Figure 2. Binding of $\alpha_1\beta_1$ to collagen IV-Sepharose. Extracts of ^{125}I surface-labeled clone HA1 cells (a–f) or CYT cells (g–l) were prepared (see MATERIALS AND METHODS) using BOG in TCM buffer (a–c and g–i) or BOG in TMM buffer (d–f and j–l). These extracts were incubated with rabbit anti-β_1 cytoplasmic peptide serum (a, d, g, and j). Immunoprecipitates were recovered by the use of protein A-Sepharose followed by analysis using SDS-PAGE. In addition, portions of the extracts were incubated with FNf-Sepharose (b, e, h, and k) or collagen IV-Sepharose (c, f, i, and l), washed, and eluted with 1 ml of a buffer containing 10 mM EDTA. These samples were then immunoprecipitated using rabbit anti-β_1 cytoplasmic peptide serum and protein A-Sepharose followed by analysis using SDS-PAGE and autoradiography. Both forms of $\alpha_1\beta_1$ specifically bind to collagen IV-Sepharose but not to FNf-Sepharose, and this interaction is dependent on Mg^{2+} and inhibited by Ca^{2+}.

Adhesive Properties of CYT and HA1 Cells

In addition to the comparison of the binding specificity between the wild-type and mutant integrins, we have examined the adhesion of CYT and HA1 cells to FN and collagen IV. Furthermore, we compared the adhesive properties of these two cell lines with those of the parental NIH 3T3 cells. Our results indicate that heterologous expression of $\alpha_1\beta_1$ in NIH 3T3 cells does not result in a major change in the adhesion to FN. Figure 3A reveals that 3T3, CYT, and HA1 cells show comparable adhesive properties on FN even though CYT and HA1 cells have the α_1 subunit as a significant portion of the integrins expressed on their surface.

In contrast, the adhesion of 3T3 cells to collagen IV is markedly altered by the expression of α_1 on their surfaces. The parental NIH 3T3 cells have minimal adhesion to collagen IV (Figure 3B). The expression of the mutant and wild-type human α_1 subunit in CYT and HA1 cells results in a functional receptor that confers the ability to adhere to collagen IV. The adherence of CYT and HA1 cells to collagen IV is similar, indicating that the deletion of the α_1 cytoplasmic domain does not markedly change the affinity of $\alpha_1\beta_1$ for collagen IV.

To further characterize the adhesion of these cells, we performed inhibition studies (Figure 3C). Complete inhibition of both CYT and HA1 cell adhesion to either FN or collagen IV was obtained with EDTA treatment, which was expected, because most integrin-mediated adhesion is divalent cation dependent. Furthermore, the anti-β_1 polyclonal serum Lenny markedly inhibited the adhesion of both cell lines to FN and collagen IV, implicating β_1-containing integrins as the primary receptors in this process. In addition, the inhibitory human α_1 monoclonal antibody (mAb) 1B3.1 ablates the adhesion of CYT and HA1 cells only to collagen IV, demonstrating that most of the adhesion to collagen IV is mediated by $\alpha_1\beta_1$. This inhibition is specific, because adhesion to FN

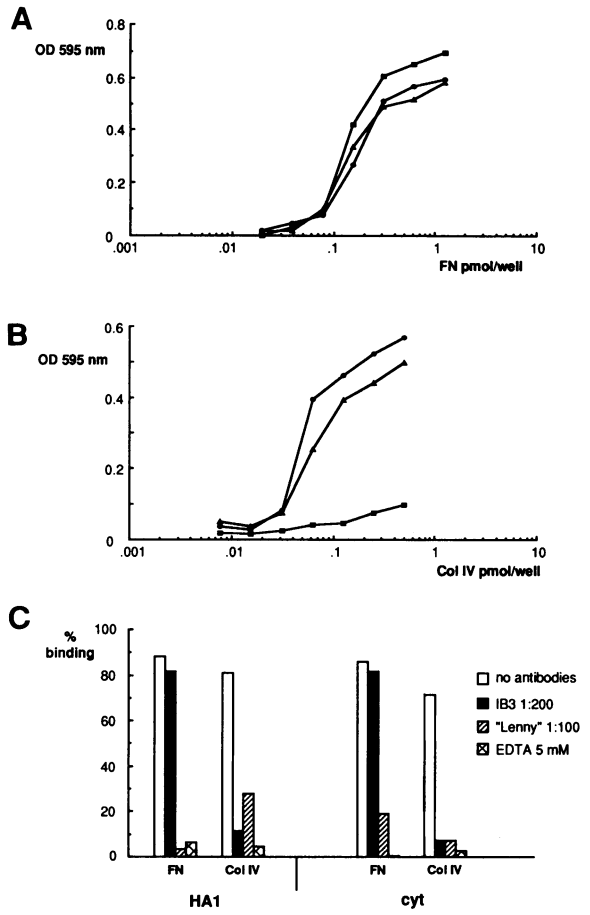


Figure 3. Adhesion assay of 3T3 cell lines. NIH 3T3 cells (squares), HA1 cells (circles), and CYT cells (triangles) were assayed for adhesion to FN (A) or to collagen IV (B). Amounts of adhesive proteins indicated at the bottom of the graphs were dried down on microtiter plates. Cells were incubated for 45 min at 37°C, washed, and the bound cells were fixed and stained. Optical density was read at 595 nm. HA1 and CYT cell lines have similar adhesion curves on FN and collagen IV. Inhibition studies are shown in C. Microtiter well surfaces were coated with FN (0.2 μg/well) or Col IV (0.1 μg/well). Cells were incubated for 30 min at 37°C in the presence of anti-human α_1 mAb 1B3.1 (black bars, final dilution 1:200), rabbit anti-rat β_1 serum Lenny (hatched bars, 1:100), 5 mM EDTA (crossed bars), or in the absence of inhibitor (open bars). Cells were fixed and then stained with crystal violet and optical density was read at 595 nm. The optical density of cells bound to an excess of FN (1 μg/well) determined the 100% value.

is unaffected by this monoclonal antibody in both cell lines.

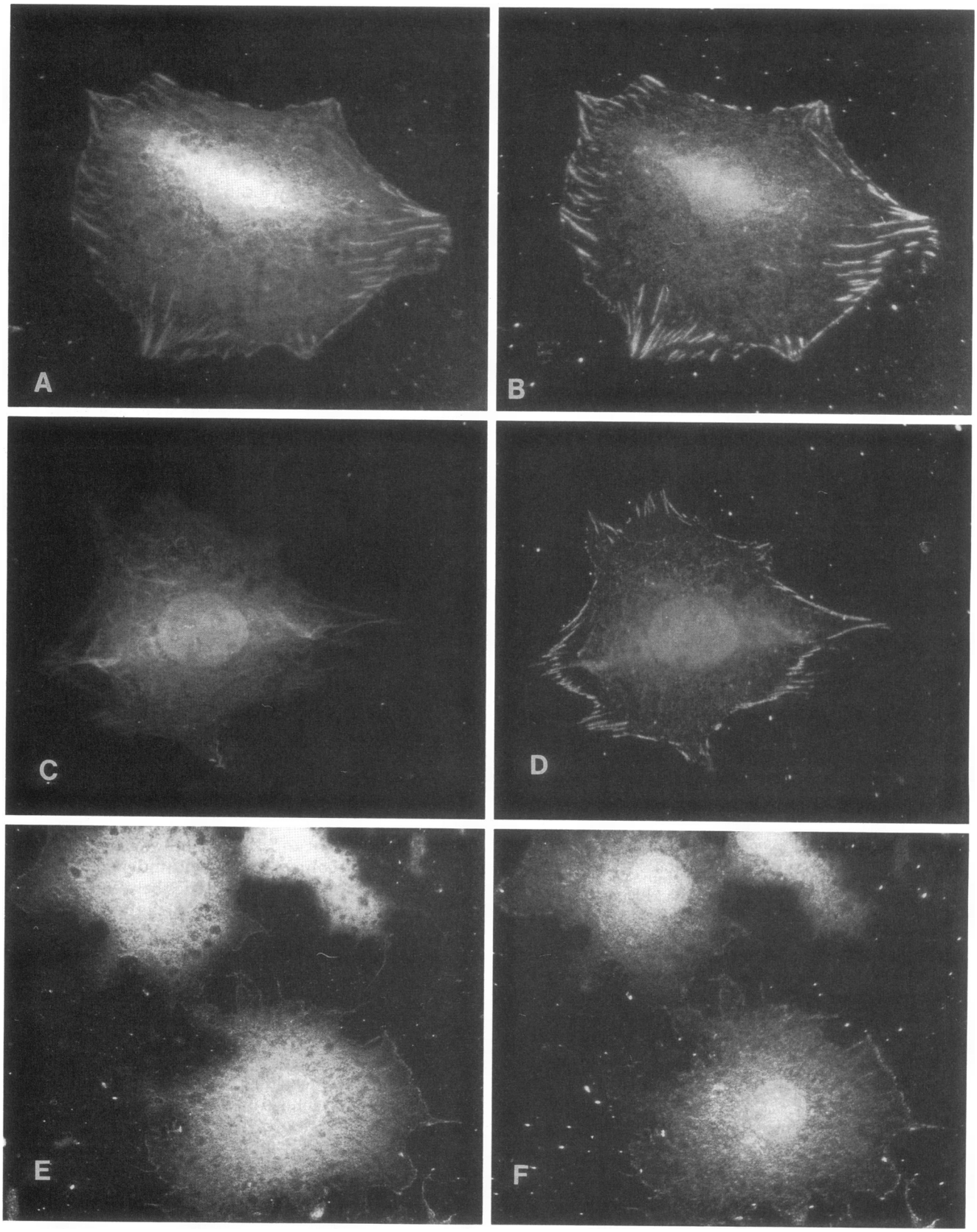
Cellular Localization of Integrin Receptors

To study the cellular localization of integrins in CYT and HA1 cells, we plated cells on collagen IV after treatment with cycloheximide to block endogenous matrix production. When CYT cells (Figure 4) are plated on collagen IV, the human α_1 subunit (Figure 4, B and D) localizes to focal contact sites as shown by colocalization with the cytoskeletal protein talin (Figure 4A). The FN receptor $\alpha_5\beta_1$ (Figure 4C) is absent from these focal contact sites. The wild-type HA1 cells behaved similarly (corroborative data). These results indicate that the focal contact localization of the endogenous FN receptor is dependent on the ligand and that endogenous FN synthesis is effectively blocked by cycloheximide treatment. Furthermore, both the wild-type and mutant human α_1 are efficiently localized to focal contact sites on their ligand, as expected. Thus, deletion of the α_1 cytoplasmic domain has no apparent effect on ligand-dependent focal contact localization.

The primary question that we sought to address was the role of the α_1 cytoplasmic domain in the prevention of ligand-independent focal contact localization. We tested this role by plating the HA1 and CYT cells on FN and performing colocalization studies for α_5 and α_1 integrin subunits with talin and vinculin as markers of focal contact sites. When HA1 cells were plated on FN, the α_5 subunit of the FN receptor (Figure 5D) localized to focal contact sites together with the cytoskeletal protein vinculin (Figure 5C). As expected, the wild-type human α_1 subunit (Figure 5, B and F) is not localized to focal contact sites indicated by α_5 (Figure 5E) or talin (Figure 5A) staining in these cells when plated on FN. Thus, for the wild-type α_1 subunit, focal contact localization is ligand dependent, because $\alpha_1\beta_1$ does not bind FN.

In contrast, when the CYT cells were plated on FN, the human α_1 subunit (Figure 6, B and F) is localized to focal contact sites as seen by the colocalization with the cytoskeletal protein talin (Figure 6A) and with the α_5 subunit (Figure 6E). As expected, vinculin and α_5 colocalize (Figure 6, C and D). Thus, the HA1 cells and CYT cells are strikingly different in the cellular localization of the α_1 subunit. Affinity chromatography and adhesion experiments have shown that the deletion of the α_1 cytoplasmic domain does not convey FN-binding affinity to the mutant $\alpha_1\beta_1$. Therefore, the mutant $\alpha_1\beta_1$ localizes to focal contacts in a ligand-independent way.

Upon seeing this striking difference between the wild-type and mutant α_1 subunits, we next performed control experiments to characterize this localization. Several studies have shown that fibroblasts will not form focal contact sites on the cell-binding fragment of FN (Woods *et al.*, 1986). We have tested both HA1 and CYT cells plated on the FN cell-binding fragment, and neither α_5 nor α_1 is localized to focal contact sites on this substrate despite adequate spreading (corroborative data). We also tested the requirement for protein kinase C (PKC) activation for both cell lines on FN and collagen IV, based on the hypothesis that this activation could be a step in the mechanism by which ligand-dependent focal adhesion localization occurs. CYT cells were plated on collagen IV with various concentration of the PKC inhibitor staurosporine (Figure 4, E and F). Focal contact site localization was markedly affected at 50 nM stau-


A
B
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D
E
F

rosporine as seen by talin staining (Figure 4E). The wild-type HA1 cells were similarly affected at this concentration (corroborative data). Thus, the deletion of the α_1 cytoplasmic domain does not lead to a fundamental change in the requirement for PKC activation in focal contact formation.

To determine whether the truncated α_1 subunit would show ligand-independent focal contact localization on a substrate other than FN, we have determined the localization of α_1 and talin on HA1 cells and CYT cells plated on VN (Figure 7). As expected, α_1 (Figure 7B) in the wild-type HA1 cells did not localize to focal contacts on VN visualized with talin staining (Figure 7A). In contrast, the truncated α_1 (Figure 7D) is colocalized with talin (Figure 7C) in focal contact sites in the CYT cells. Once again, the truncated α_1 subunit localized to focal contacts in a ligand-independent manner.

DISCUSSION

In this study, we have deleted the cytoplasmic domain of the α_1 integrin subunit to investigate whether it has an effect on the focal contact localization signal in the β_1 subunit. Two NIH 3T3 cell lines were created that express the human α_1 wild-type cDNA (HA1 cells) and the cytoplasmic domain deleted α_1 cDNA (CYT cells). Immunoprecipitation experiments showed that both cell lines express similar levels of the human α_1 subunit and that there is no significant effect of the deletion on the dimerization and surface expression of the receptor. Furthermore, both affinity chromatography and cell adhesion experiments show that ligand specificity remained unchanged despite this deletion. However, when these two cell lines are plated on FN, which is a ligand for $\alpha_5\beta_1$ and not for $\alpha_1\beta_1$ in these cells, there is a striking difference between the two. The HA1 cells show only α_5 in focal contacts, without α_1. This is expected because $\alpha_1\beta_1$ does not bind to FN; therefore, all of the integrin localization is ligand dependent. In the wild-type α_1/mouse β_1 receptor, there may be an inhibitory component that prevents the β_1 cytoplasmic domain from directing this heterodimer to focal contact sites. In contrast, when the CYT cells are plated on FN, the mutant α_1/mouse β_1 is directed to focal contacts along with the α_5/β_1. Thus, there is both ligand-dependent (α_5/β_1) and ligand-independent (α_1/β_1) focal contact localization in these cells. The mutant α_1 has apparently lost the inhibitory aspect of the focal contact localization, and we conclude that the β_1 cytoplasmic domain is constitutively active in this receptor as it is in the interleukin/β_1 (LaFlamme *et al.*, 1992) and N-cadherin/β_1 chimeras (Geiger *et al.*, 1992). This ligand-independent localization appears to be as vigorous as the ligand-dependent localization of either the wild-type or mutant $\alpha_1\beta_1$ on collagen IV.

One possible explanation for these results would be that the mutant has acquired the ability to bind to FN. We have done several experiments that make that conclusion unlikely. Affinity chromatography experiments show that the mutant and wild-type α_1/β_1 receptors do not bind to FNf-Sepharose, and binding to collagen IV-Sepharose is very similar between the two receptors. Furthermore, adhesion of the two cell lines to FN is very similar to the parental 3T3 line, suggesting that similar integrins are used. Despite an increased number of integrin receptors ($\alpha_1\beta_1$) on the cell surface, HA1 or CYT cells have a similar binding curve on FN as the parental NIH 3T3 cells (Figure 3). Addition of an α_1 blocking antibody (1B3.1) has no effect on the adhesion of either cell line to FN, while effectively blocking adhesion to collagen IV (Figure 4) or laminin (corroborative data). Finally, because the adhesion of $\alpha_1\beta_1$ to collagen and laminin is dependent on Mg^{2+}, we have compared the adhesion of 3T3, HA1, and CYT cells with FN in the presence of Ca^{2+} or Mg^{2+} and found that it was identical in either cation (corroborative data). Taken together, these experiments suggest that it is very unlikely that the truncated α_1 binds to FN.

In addition to the control experiments described above, we have also tested the localization of wild-type and truncated α_1 on VN. As for FN, wild-type α_1 did not localize to focal contacts, whereas the truncated form did. Formation of focal contacts is not very vigorous on this substrate in 3T3 cells, presumably because of the small amount of $\alpha_v\beta_3$ present on these cells. We think it is very unlikely that the truncated α_1 binds to VN, again showing ligand-independent focal contact localization.

The deletion of the α_1 cytoplasmic domain allows the mutant $\alpha_1\beta_1$ receptor to localize in focal contacts organized by a wild-type integrin like $\alpha_5\beta_1$ on FN. We have also tested whether this mutant α subunit could organize focal contacts de novo in a ligand-independent manner. When CYT cells are plated on polylysine or the 120-kDa fragment of FN, no focal contact formation is observed (corroborative data). The 120-kDa fragment has been shown to promote cell adhesion via $\alpha_5\beta_1$, but focal

Figure 4. Double-label immunofluorescence of cells expressing the truncated α_1 subunit on collagen IV. The CYT cells were plated on collagen IV and stained with a mixture of rabbit anti-talin and mouse monoclonal anti-human α_1 TS2/7 (A and B) and rabbit anti-α_5 and mouse monoclonal anti-human α_1 TS2/7 (C and D). The primary antibodies were visualized by the use of rhodamine-conjugated rabbit anti-mouse IgG (B, D, and F) and fluorescein-conjugated goat anti-rabbit IgG (A, C, and E). Despite the loss of the α_1 cytoplasmic domain, focal contact formation of $\alpha_1\beta_1$ on collagen IV (ligand dependent) is identical to the wild-type integrin. In E and F, CYT cells were plated on collagen IV in the presence of 50 nM staurosporine and stained with a mixture of rabbit anti-talin (E) and mouse monoclonal anti-human α_1 TS2/7 (F). There is a marked loss of focal contact formation after staurosporine treatment.

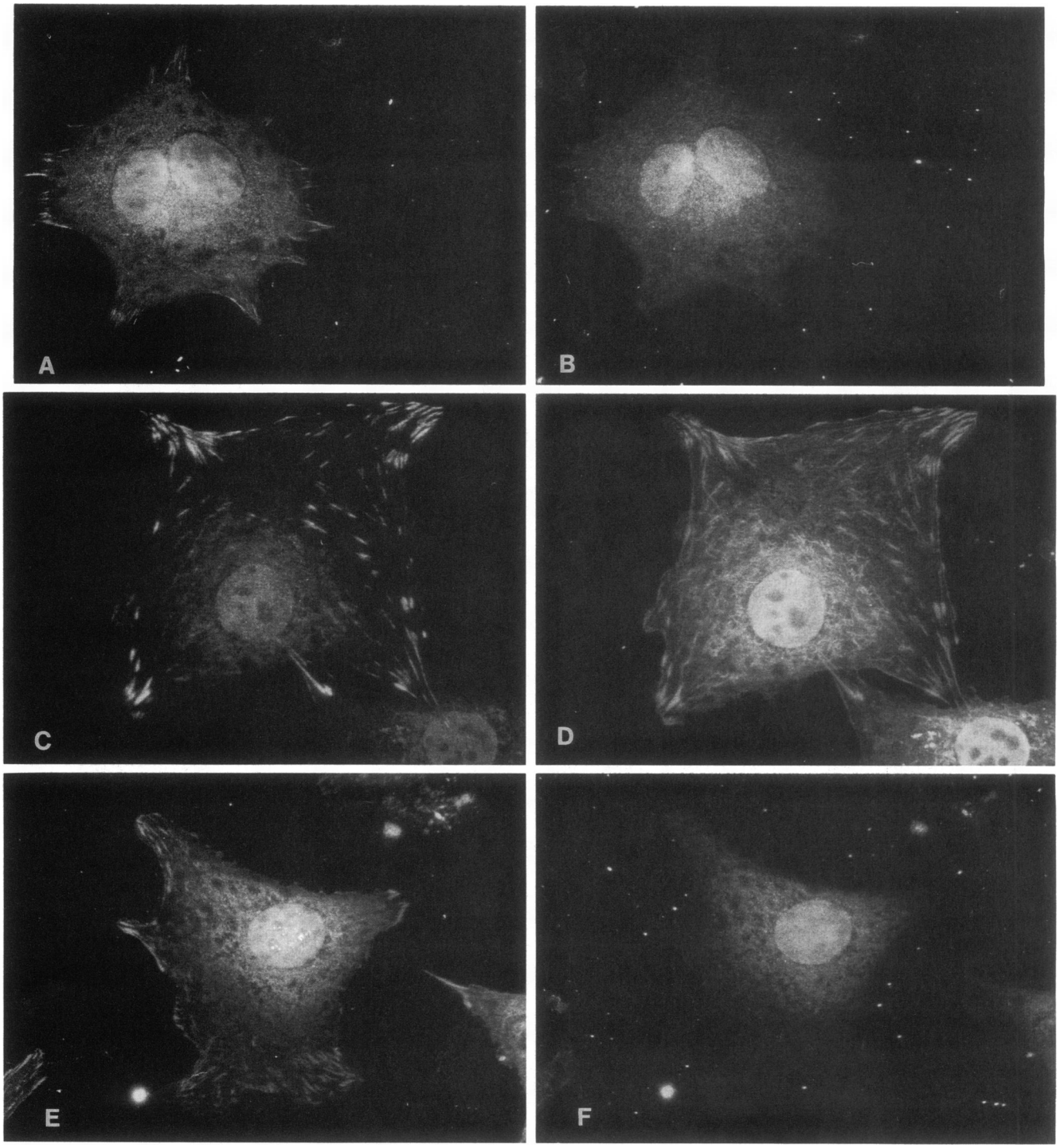

Figure 5. Double-label immunofluorescence of cells expressing the wild-type α_1 subunit on FN. HA1 cells were plated on fibronectin and stained with a mixture of rabbit anti-talin and mouse monoclonal anti-human α_1 TS2/7 (A and B), mouse anti-vinculin and rabbit anti-α_5 (C and D), and rabbit anti-α_5 and mouse monoclonal anti-human α_1 TS2/7 (E and F). The primary antibodies were visualized by the use of rhodamine-conjugated rabbit anti-mouse IgG (B, C, and F) and fluorescein-conjugated goat anti-rabbit IgG (A, D, and E). The wild-type α_1 does not localize to focal contacts defined by α_5 and talin staining.

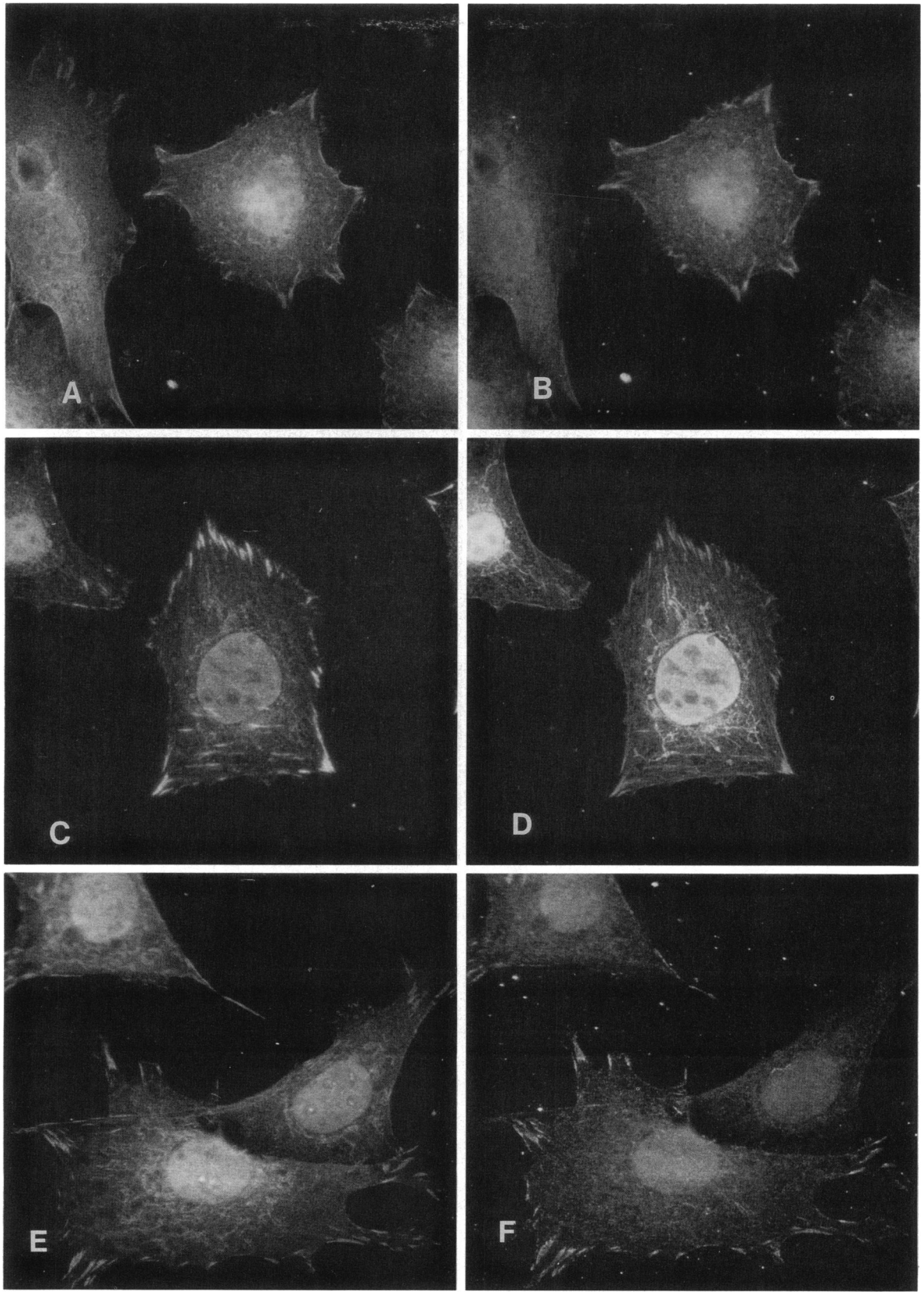

Figure 6. Double-label immunofluorescence of cells expressing the truncated α_1 subunit on FN. CYT cells were plated on fibronectin and stained with a mixture of rabbit anti-talin and mouse monoclonal anti-human α_1 TS2/7 (A and B), mouse anti-vinculin and rabbit anti-α_5 (C and D), and rabbit anti-α_5 and mouse monoclonal anti-human α_1 TS2/7 (E and F). The primary antibodies were visualized by the use of rhodamine-conjugated rabbit anti-mouse IgG (B, C, and F) and fluorescein-conjugated goat anti-rabbit IgG (A, D, and E). Note that the mutant α_1 colocalizes with talin and α_5 in focal contacts in a ligand-independent manner.

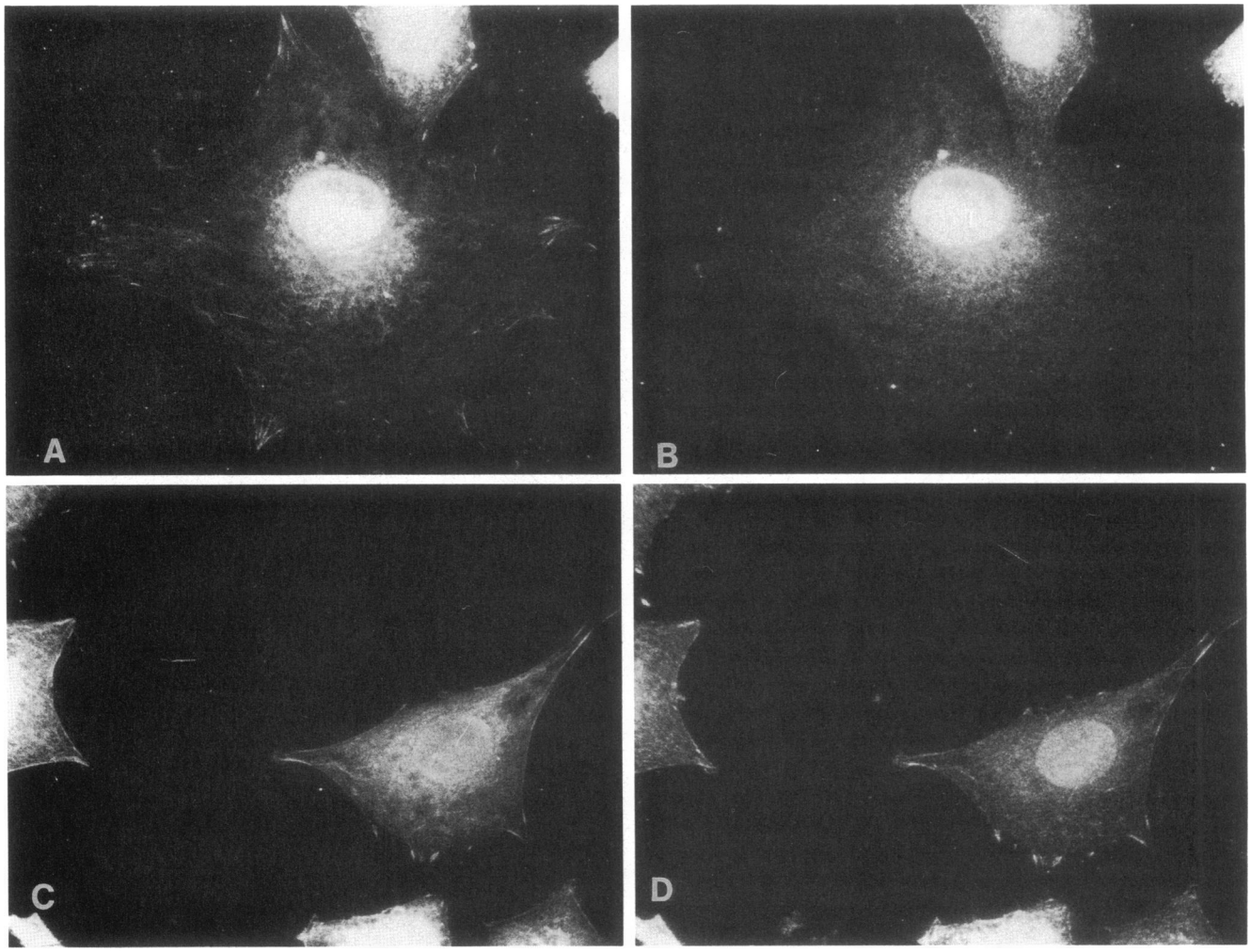

Figure 7. Double-label immunofluorescence of cells expressing either the wild-type or the truncated α_1 subunit on VN. HA1 cells (A and B) or CYT cells (C and D) were plated on vitronectin and stained with a mixture of rabbit anti-talin and mouse monoclonal anti-human α_1 TS2/7 (A and B). The primary antibodies were visualized by the use of rhodamine-conjugated rabbit anti-mouse IgG (B and D) and fluorescein-conjugated goat anti-rabbit IgG (A and C). Note that the mutant α_1 colocalizes with talin in focal contacts in a ligand-independent manner.

contact formation requires the heparin-binding fragment of FN (Woods *et al.*, 1986). As we have shown, $\alpha_1\beta_1$ in CYT cells behaves as if it has bound ligand, that is, the β_1 cytoplasmic domain presumably is in an "active" state. However, this active state is not sufficient to promote the aggregation of $\alpha_1\beta_1$ and the nucleation of focal contacts de novo without ligand. Presumably, the ligand provides a clustering mechanism necessary for focal contact organization in addition to providing the signal that produces the active state of the β_1 cytoplasmic domain.

The molecular events required to initiate integrin focal contact localization are not well understood. Recent studies by Woods and Couchman (1992) have shown that PKC activation is required for the initiation of focal contact sites. We hypothesized that the PKC-dependent event could be activation of the β_1 cytoplasmic domain, an event similar to the loss of the α_1 cytoplasmic domain inhibitory signal. Because we have seen a loss in ligand requirement for focal contact localization in the CYT cell line, we compared the effect of PKC inhibitors on ligand-dependent or -independent focal contact formation. When either staurosporine or down-regulation by long-term treatment with phorbol esters was used to inhibit PKC function, there was marked depletion of focal contact formation on FN in these cells. PKC inhibition prevented focal contact localization on collagen IV for both cell lines. Thus, we conclude that the PKC-dependent event required for integrin focal contact localization is not activation of the β_1 cytoplasmic domain. In fact, staurosporine begins to affect the shape of the cell before abolishing focal adhesion formation, suggesting that the major effect is on the cytoskeleton.

Recently, Takada *et al.* (1992) reported a point mutation in the extracellular domain of the β_1 subunit that abolishes ligand binding. Strikingly, this mutant β_1 will localize to focal contact sites along with the endogenous wild-type β_1 on FN in an apparently ligand-independent manner. Presumably, this point mutation causes a conformational change similar to the change triggered by bound ligand, leading to an active β_1 cytoplasmic domain.

Recently, there have been a number of studies published that investigate the function of integrin α subunit cytoplasmic domains. Expression of the platelet fibrinogen receptor $\alpha_{IIb}\beta_3$ in Chinese hamster ovary (CHO) cells leads to a latent receptor that can be activated with certain anti-receptor antibodies (O'Toole *et al.*, 1990). This is similar to the situation in platelets where activation via physiological mediators is required for activity. Expression of an α_{IIb} without its cytoplasmic domain in CHO cells leads to a constitutively active receptor (O'Toole *et al.*, 1991), and substitution of the α_5 cytoplasmic domain for the native domain will not suppress this activity. In another system, Chan *et al.* (1992) have shown that the exchange of α cytoplasmic domain can change the functions of the respective integrins. These authors made chimeras of the extracellular portion of the integrin α_2 and various α subunit cytoplasmic domains. When the α_5 cytoplasmic domain was substituted for the α_2 cytoplasmic domain, collagen gel contraction via $\alpha_2\beta_1$ was unaffected. However, when the α_4 cytoplasmic domain was used, cells migrated on collagen but would not contract the gel. One explanation for these results might be that these different α cytoplasmic domains had differential effects on the "exposure" or "activation" state of the β_1 cytoplasmic domain.

We have shown that one function of the α_1 cytoplasmic domain may be the regulation of focal contact localization of $\alpha_1\beta_1$. The cytoplasmic domains of other α subunits may play a similar role. Despite many differences in the amino acid sequences between various α cytoplasmic domains, a common mechanism of interaction between α and β cytoplasmic domains could exist. In future experiments, we hope to determine which amino acids are responsible for the effect of the α_1 cytoplasmic domain on ligand-independent focal contact localization. Such knowledge would be useful in understanding the effects of phosphorylation (e.g., Buyon *et al.*, 1990; Shaw *et al.*, 1990) or alternative splicing of α cytoplasmic domains (Hogervorst *et al.*, 1991; Tamura *et al.*, 1991) on integrin function.

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REFERENCES

Albelda, S.M., Daise, M., Levine, E.N., and Buck, C.A. (1989). Identification and characterization of cell substratum adhesion receptors on cultured human adult large vessel endothelial cells. J. Clin. Invest. *83*, 992–1002.

Aumailley, M., Mann, K., von der Mark, H., and Timpl, R. (1989). Cell attachment properties of collagen type VI and Arg-Gly-Asp dependent binding to its α2 (VI) and α3 (VI) chains. Exp. Cell Res. *181*, 463–474.

Bank, I., Hemler, M., Brenner, M.B., Cohen, D., Levy, V., Belko, J., Crouse, C., and Chess, L. (1989). A novel monoclonal antibody, 1B3.1, binds to a new epitope of the VLA-1 molecule. Cell. Immunol. *122*, 416–423.

Briesewitz, R., Epstein, M.R., and Marcantonio, E.E. (1993). The expression of native and truncated forms of the human integrin α_1 subunit. J. Biol. Chem. *268*, 2989–2996.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988). Focal adhesion: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell Biol. *4*, 487–525.

Buyon, J.P., Slade, S.G., Reibman, J., Abramson, S.B., Philips, M.R., Weissman, G., and Wincester, R. (1990). Constitutive and induced phosphorylation of the α and β chains of the CD11/CD18 leukocyte integrin family. Relationship to adhesion-dependent functions. J. Immunol. *144*, 191–197.

Chan, B.M.C., 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 α subunit cytoplasmic domains. Cell *68*, 1051–1060.

Chen, W.T., Hasegawa, C., Weinstock, C., and Yamada, K.M. (1985). Development of cell surface linkage complexes in cultivated fibroblasts. J. Cell Biol. *100*, 1103–1114.

Damsky, C.M., Knudsen, K.A., Bradley, D., Buck, C.A., and Horwitz, A.F. (1985). Distribution of the CSAT cell-matrix antigen on myogenic and fibroblastic cells in culture. J. Cell Biol. *100*, 1528–1539.

Dejana, E., Colella, S., Conforti, G., Abbadini, M., Gaboli, M., and Marchisio, P.C. (1988). Fibronectin and vitronectin regulate the organization of their respective Arg-Gly-Asp adhesion receptors in cultured human endothelial cells. J. Cell Biol. *107*, 1215–1223.

DeSimone, D.W., and Hynes, R.O. (1988). *Xenopus laevis* integrins: structural conservation and evolutionary divergence of integrin β subunits. J. Biol. Chem. *263*, 5333–5340.

Engvall, E., and Ruoslahti, E. (1977). Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer *20*, 201–205.

Geiger, B., Salomon, D., Takeichi, M., and Hynes, R.O. (1992). A chimeric N-cadherin/β_1-integrin receptor which localizes to both cell-cell and cell-matrix adhesions. J. Cell Sci. *103*, 943–951.

Hall, D.E., Reichardt, L.F., Crowley, E., Holley, B., Moezzi, H., Sonnenberg, A., and Damsky, C.H. (1990). The $\alpha_1\beta_1$ and $\alpha_6\beta_1$ integrin heterodimers mediate cell attachment to distinct sites on laminin. J. Cell Biol. *110*, 2175–2184.

Hayashi, Y., Haimovich, B., Rezka, A., Boettiger, D., and Horwitz, A.F. (1990). Expression and function of chicken integrin β_1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. J. Cell Biol. *110*, 175–184.

Hemler, M.E. (1990). VLA proteins in the integrin family: structures, functions, and their role on leukocytes. Annu. Rev. Immunol. *8*, 365–400.

Hogervorst, F., Kuikman, I., van Kessel, A.G., and Sonnenberg, A. (1991). Molecular cloning of the human α_6 integrin subunit. Alternative splicing of the α_6 mRNA and chromosomal localization of the α_6 and β_4 genes. Eur. J. Biochem. *199*, 425–433.

Horwitz, A.F., Duggan, E., Buck, C., Beckerle, M.C., and Burridge, K. (1986). Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. Nature *320*, 531–533.

Hynes, R.O. (1973). Alteration of cell-surface proteins by viral transformation and by proteolysis. Proc. Natl. Acad. Sci. USA *70*, 3170–3174.

Hynes, R.O. (1992). Integrins: versatility, modulation and signalling in cell adhesion. Cell *69*, 11–25.

Hynes, R.O., Marcantonio, E.E., Stepp, M.A., Urry, L.A., and Yee, G.E. (1989). Integrin heterodimer and receptor complexity in avian and mammalian cells. J. Cell Biol. *109*, 409–420.

Kramer, R.H., and Marks, N. (1989). Identification of integrin collagen receptors on human melanoma cells. J. Biol. Chem. *264*, 4684–4688.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature *227*, 680–685.

LaFlamme, S.E., Akiyama, S.K., and Yamada, K.M. (1992). Regulation of fibronectin receptor distribution. J. Cell Biol. *117*, 437–447.

Marcantonio, E.E., Guan, J-I., Trevethick, J.E., and Hynes, R.O. (1990). Mapping of the functional determinants of the integrin β_1 cytoplasmic domain by site directed mutagenesis. Cell Regul. *1*, 597–604.

Marcantonio, E.E., and Hynes, R.O. (1988). Antibodies to the conserved cytoplasmic domain of the integrin β_1 subunit react with proteins in vertebrates, invertebrates and fungi. J. Cell Biol. *106*, 1765–1772.

O'Toole, T.E., Loftus, J.C., Du, X.P., Glass, A.A., Ruggeri, Z.M., Shattil, S.J., Plow, E.F., and Ginsberg, M.H. (1990). Affinity modulation of the $\alpha_{IIb}\beta_3$ integrin is an intrinsic property of the receptor. Cell Regul. *1*, 883–893.

O'Toole, T.E., Mandelman, D., Forsyth, J., Shattil, S.J., Plow, E.F., and Ginsberg, M.H. (1991). Modulation of the affinity of integrin $\alpha_{IIb}\beta_3$ (GP IIb-IIIa) by the cytoplasmic domain of α_{IIb}. Science *254*, 845–847.

Otey, C.A., Pavalko, F.M., and Burridge, K. (1990). An interaction between α-actinin and the β_1 integrin subunit in vitro. J. Cell Biol. *111*, 721–729.

Reszka, A.A., Hayashi, Y., and Horwitz, A.F. (1992). Identification of amino acid sequences in the integrin β_1 cytoplasmic domain implicated in cytoskeletal association. J. Cell Biol. *117*, 1321–1329.

Shaw, L.M., Messier, J.M., and Mercurio, A.M. (1990). The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the $\alpha_6\beta_1$ integrin. J. Cell Biol. *110*, 2167–2174.

Singer, I.I., Scott, S., Kawka, D.W., Kazazis, D.M., Gailit, J., and Rouslahti, E. (1988). Cell surface distribution of fibronectin and vitronectin receptor depends on substrate composition and extracellular matrix accumulation. J. Cell Biol. *106*, 2172–2182.

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.

Southern, P.J., and Berg, P. (1982). Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. *1*, 327–341.

Springer, T.A. (1990). Adhesion receptors of the immune system. Nature *346*, 425–434.

Takada, Y., Ylänne, J., Mandelman, D., Puzon, W., and Ginsberg, M.H. (1992). A point mutation of integrin β_1 subunit blocks binding of $\alpha_5\beta_1$ to fibronectin and invasin but not recruitment to adhesion plaques. J. Cell Biol. *119*, 913–921.

Tamura, R.N., Rozzo, C., Starr, L., Chambers, J., Reichardt, L.F., Cooper, H.M., and Quarenta, V. (1991). Cell-type specific integrin variants with alternative α chain cytoplasmic domains. Proc. Natl. Acad. Sci. USA *88*, 10183–10187.

Tapley, P., Horwitz, A.F., Buck, C.A., Burridge, K., Duggan, K., Hirst, R., and Rohrschneider, L. (1989). Analysis of the avian fibronectin receptor (integrin) as a direct substrate for pp60 v-src. Oncogene *4*, 325–333.

Turner, D.C., Flier, L.A., and Carbonetto, S. (1989). Identification of a cell surface protein involved in PC12 cell-substratum adhesion and neurite outgrowth on laminin and collagen. J. Neurosci. *9*, 3287–3296.

Vandenberg, P., Kern, A., Ries, A., Luckenbill-Edds, L., Mann, K., and Kühn, K. (1991). Characterization of a type IV collagen major cell binding site with affinity to the alpha 1 beta 1 and the alpha 2 beta 1 integrins. J. Cell Biol. *113*, 1475–1483.

Woods, A., and Couchman, J.R. (1992). Protein kinase C involvement in focal adhesion formation. J. Cell Sci. *101*, 277–290.

Woods, A., Couchman, J.R., Johansson, S., and Höök, M. (1986). Adhesion and cytoskeletal organization of fibroblasts in response to fibronectin fragments. EMBO J. *5*, 665–670.