The Membrane–Cytoplasm Interface of Integrin α Subunits Is Critical for Receptor Latency

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Localization of integrin receptors to focal contact sites occurs upon ligand binding. This activity is latent, since unoccupied integrin receptors do not localize to focal contacts. Deletion analysis has revealed that the α cytoplasmic domain is required for the maintenance of integrin receptor latency. Our current hypothesis for the mechanism of integrin post-ligand binding events is that there is a change in relationship of α and β cytoplasmic domains, which overcomes receptor latency. One possible mechanism for such a change would involve the amino acid residues at the membrane–cytoplasm interface. To test this hypothesis, we have produced point mutations in the human integrin α_1 subunit. These mutations had no effect on the adhesion via $\alpha_1\beta_1$ to its ligand, collagen IV. However, receptor latency is lost in one of these mutants, leading to constitutive focal contact localization. This effect did not occur in receptors with an exchange of intracellular domains, suggesting that the mechanism of loss of latency involves a relative motion of the integrin chains. These results suggest a model in which post-ligand binding events in integrin receptors are associated with changes in the position of the α and β cytoplasmic domains.

INTRODUCTION

Integrin receptors each consist of α and β subunits that associate to mediate interactions between cells and the extracellular matrix (ECM). These receptors often concentrate in specific regions within the plasma membrane, called focal contact sites, after having bound their ECM substrates (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 integrin β_1 subunit in vitro, suggesting a direct interaction in vivo (Horwitz *et al.*, 1986; Otey *et al.*, 1990). Thus, integrins are thought to provide a mechanical linkage between the ECM and the cytoskeleton.

In addition, it is now clear that integrins are involved in providing attachment-dependent growth signals. Adhesion is required for normal cell cycle progression (Guadagno and Assoian, 1991), and attachment to fibronectin can provide the signals necessary for progression (Guadagno *et al.*, 1993). Furthermore, this attachment leads to MAP kinase activation (Chen *et al.*, 1994; Zhu and Assoian, 1995), presumably through activation of ras. Integrin attachment to the cytoskeleton seems to be required for these signals, because all of these events can be disrupted by cytochalasin D treatment (Zhu and Assoian, 1995). Thus, it seems that interaction with the cytoskeleton at the focal adhesion may be the initial step in integrin receptor function.

All integrin subunits have carboxyl-terminal cytoplasmic domains that are usually short. The β_1 cytoplasmic domain is required for localization to focal adhesions (Solowska *et al.*, 1989; Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990; Reszka *et al.*, 1992), and this domain is sufficient to direct reporter constructs to

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focal adhesions (Geiger *et al.*, 1992; LaFlamme *et al.*, 1992). Expression of these reporter constructs in cells leads to a disruption of a number of integrin receptor functions, including migration, matrix assembly, and tyrosine phosphorylation (LaFlamme *et al.*, 1994; Lukashev *et al.*, 1994). In NIH-3T3 cells containing a version of these constructs, stimulation of the actin cytoskeleton via lysophosphatidic acid (LPA)¹ leads to a rapid detachment of cells from the substratum (Smilenov *et al.*, 1994). These results are consistent with the idea that the β cytoplasmic domain is the major link with the actin cytoskeleton.

Work by a number of laboratories has shown that the α cytoplasmic domain plays a key role in integrin receptor function. Unoccupied integrin receptors do not localize to focal adhesions (Dejana *et al.*, 1988; Singer *et al.*, 1988) but are rapidly recruited to pre-formed focal adhesions upon binding of ligand (Laflamme *et al.*, 1992). Deletions of the distal α cytoplasmic domains in a number of integrin receptors lead to constitutive or ligand-independent focal contact localization (Briesewitz *et al.*, 1993b; Ylännä *et al.*, 1993; Kawaguchi *et al.*, 1994). Thus, the α cytoplasmic domain is required for integrin receptor latency.

Experiments from our laboratory have shown that apposition of α and β cytoplasmic domains is sufficient to maintain integrin receptor latency (Briesewitz *et al.*, 1995). These results led to a model that suggests that α and β cytoplasmic domains may associate during subunit assembly and that this association is perturbed upon ligand binding, leading to β cytoplasmic domain mediation of post-ligand binding events. One mechanism that has been proposed for such conformation changes would involve the membrane-cytoplasm interface of integrin subunits (Williams *et al.*, 1994). We have designed experiments to test the role of the amino acids at the membrane-cytoplasm interface via site-directed mutagenesis. We now show that this region is involved in the normal maintenance of integrin receptor latency.

MATERIALS AND METHODS

Site-directed Mutagenesis and Plasmid Construction

Restriction enzymes and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA), T4 DNA ligase from Life Technologies (Gaithersburg, MD), and *Taq* polymerase from Boehringer-Mannheim (Indianapolis, IN). Oligonucleotides were purchased from Operon Technologies (Alameda, CA) and were used directly without further purification.

Point mutations A³⁴⁹⁸AG to G³⁴⁹⁸AT and A³⁴⁹⁸AG to G³⁴⁹⁸CT leading to ¹¹³⁷Lys-¹¹³⁷Asp and ¹¹³⁷Lys-¹¹³⁷Ala amino acid substitution, respectively, within the human α_1 sequence (Briesewitz *et al.*, 1993a) were introduced by using synthetic oligonucleotides and overlapping PCR and blunt-end cloned into pBluescript, followed

by subcloning into the human α_1 cDNA in the expression vector pLEN as a *Bam*HI fragment. The fidelity of all regions amplified by polymerase chain reaction (PCR) was verified by dideoxy sequencing.

The construction of the chimeric cDNAs $\beta x\alpha c$ and $\alpha x\beta c$ has been described previously (Briesewitz *et al.*, 1995). For the production of the cDNA $\beta x\alpha c$ K>D, a point mutation, A³⁴⁹⁸AG to G³⁴⁹⁸AT, leading to ¹¹³⁷Lys-¹¹³⁷Asp amino acid substitution within the $\beta x\alpha c$ was introduced by using synthetic oligonucleotides and overlapping PCR and blunt-end cloned into pBluescript. A *Bgl*II-*Xba* fragment was subsequently subcloned into pLEN β_1 .

DNA Transfections

NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (CS). Plated the previous day, 5×10^5 cells were cotransfected with 10–20 μ g of the various chimeric cDNAs in the pLEN expression vector system (Marcantonio *et al.*, 1990) and with 2 μ g of pSV2 neo (Southern and Berg, 1982) for the primary transgene or pSV HIS-D (Hartman and Mulligan, 1988) for the second transgene in cells already neo-resistant. The calcium phosphate transfection method was used as previously described (Solowska *et al.*, 1989). After 3 d, the cells were split into DMEM with 10% CS containing 1 mg/ml G418 (Geneticin; Life Technologies, Gaithersburg, MD) in the case of the first transgene or 3 mM histidinol (Sigma Chemical, St. Louis, MO)/0.5 mg/ml G418 for the second transgene. After 10–14 d, resistant clones were isolated and screened by immunoprecipitation. For each construct, multiple clones were isolated and characterized. Although the data shown are usually from one clone, similar results have been seen with the other clones as well. 3T3 IL2R β_1 cells were produced and characterized as described previously (Smilenov *et al.*, 1994).

Antibodies

Rabbit anti- β_1 cytoplasmic domain antibodies were prepared as described (Marcantonio and Hynes, 1988). Monoclonal anti-human α_1 (TS2/7) was a gift of T. Springer (Harvard Medical School, Boston, MA). The rabbit anti-talin serum was a kind gift of K. Burridge (University of North Carolina, Chapel Hill, NC).

Radiolabeling and Immunoprecipitation

Cells were labeled with Na ¹²⁵I (New England Nuclear, Boston, MA) and lactoperoxidase as a monolayer, as described (Hynes, 1973). Per experiment, 10⁷ cells and 1–2 mCi/ml were used. The cells were extracted with 0.5% NP-40 and immunoprecipitated as described (Marcantonio and Hynes, 1988). SDS-PAGE was performed by the method of Laemmli (1970). Separation gels were 7% acrylamide with a 3% stacking gel. Samples were prepared in sample buffer (5% SDS, 100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 10% glycerol, and bromophenol blue) and boiled for 3 min.

Immunofluorescence

Solutions of 0.5 μ g of either fibronectin (FN) or the CB3 fragment of human collagen type IV in 70 μ l of water were air dried onto coverslips. After drying, the proteins were rehydrated in phosphate-buffered saline (PBS) for 30 min. Cells were treated with 25 μ g/ml cycloheximide for 2 h before trypsinization. The trypsinized cells were washed twice in PBS and plated onto the coated glass coverslips in serum-free media plus cycloheximide. The cells were then placed in a tissue culture incubator at 37°C for 3 h before fixing them in 4% paraformaldehyde in PBS for 5 min and permeabilizing them with 0.2% NP-40 in PBS for 5 min afterward. The antibody against talin was diluted 1:400 in 10% normal goat serum in PBS. TS2/7 hybridoma supernatant was supplemented with normal goat serum (10% final) and then used directly. The coverslips were overlaid with 20 μ l of primary antibody solution and incubated at 37°C for 30 min. After washing with PBS, the coverslips were

¹ Abbreviations used: FN, fibronectin; LPA, lysophosphatidic acid; PBS, phosphate-buffered saline.

incubated with 20 μ l of secondary antibody for 30 min at 37°C. The secondary antibodies were F(ab')₂ fragments of fluorescein-conjugated rabbit anti-mouse immunoglobulin G (IgG) and rhodamine-conjugated goat anti-rabbit IgG (Cappel, Durham, NC), diluted 1:250 in 10% normal goat serum in PBS.

Cell Adhesion Assays

Adhesion assays were done essentially as described previously (Kern *et al.*, 1994). 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 being washed with PBS, the remaining protein binding sites were blocked with bovine serum albumin (BSA; 1% in PBS). Cells were resuspended at $4-5 \times 10^5$ cells/ml in serum-free DMEM or adhesion medium (25 mM HEPES, pH 7.1, 150 mM NaCl, 3 mM KCl, 0.45% glucose, 0.2% heat-inactivated BSA, 1 mM MgCl₂ or 1 mM CaCl₂) and incubated in the wells for 30 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. All experiments were done in triplicate.

For inhibition assays, cells were preincubated for 10 min at room temperature in adhesion medium with a 1:200 dilution of inhibitory mouse anti-human α_1 antibody 1B3.1 and then transferred to wells coated with collagen IV (0.01 μ g/well). After 30 min at 37°C, non-adherent cells were removed, and bound cells were fixed and detected as described above. All experiments were done in triplicate.

To assay the effect of LPA, we diluted FN (1 μ g/cover slip) in distilled water and allowed it to dry on glass coverslips at room temperature. Cells were suspended in complete medium and incubated on the coverslips for 16 h. LPA (5 μ g/ml; Sigma Chemical) was added to the cells for 5 min.

Flow Cytometry

Fluorescence flow cytometric analysis was performed on a FACS-scan cytofluorometer (Becton Dickinson, Mountain View, CA). The cells were trypsinized and washed with growth media. The cells were resuspended in PBS with 0.5% CS and 0.1% NaN₃ by using 5×10^5 cells in 50 μ l. Staining was performed in primary antibody on ice for 30 min, followed by three washes with cold PBS. Cells were then resuspended and stained with FITC-conjugated goat anti-mouse antibody (TAGO) 1:100 dilution for 30 min. After three washes, cells were analyzed.

RESULTS

Expression of Mutant Human α_1 Subunits Results in a Functional Collagen IV Receptor

To study the role of the membrane-proximal region of integrin α subunits, we have introduced point mutations into the human α_1 subunit via site-directed mutagenesis. The mutations we elected to study include a substitution of lysine 1137 with an aspartate residue (K>D) and a substitution of the same lysine residue with an alanine (K>A). This lysine lies within a consensus sequence highly conserved among all integrin α subunits (see Table 1 for sequences of mutants). Both mutant segments were cloned into the full-length human α_1 cDNA. To study the localization and adhesive properties of these mutants, we cotransfected into mouse NIH-3T3 cells the mutant human α_1 cDNA in an expres-

Table 1. Mutations of α cytoplasmic domain

HA1	<u>W-K-I-G-F-F-K-R-P-L-K-K-K-M-E-K-COOH</u>
HA1 K>A	<u>W-A-I-G-F-F-K-R-P-L-K-K-K-M-E-K-COOH</u>
HA1 K>D	<u>W-D-I-G-F-F-K-R-P-L-K-K-K-M-E-K-COOH</u>

The underlined sequences represent the junction between the membrane and the cytoplasm of the integrin α_1 and fits the consensus sequence for integrin α subunits (W/Y)-(K/R)-X-G-F-F-K-R, where X is a hydrophobic amino acid. The bolded residues are those mutated.

sion vector along with a plasmid conferring neomycin resistance and selected positive clones. ¹²⁵I-surface labeling experiments followed by immunoprecipitation show that the mutant subunits dimerize with mouse β_1 and are efficiently expressed on the surface (Figure 1, panel A). In addition, flow cytometry measurements show similar levels of α_1 on the cell surfaces (Figure 1, panel B).

We have compared the adhesive capacity of mutant cell lines with that of HA1 3T3 cells, which express high levels of the wild-type human α_1 subunit (Briesewitz *et al.*, 1993a,b). In Figure 2A, we have measured the adhesion of three α_1 -containing cell lines and compared them with parental 3T3 cells by using collagen IV as a ligand. As we have shown previously, parental 3T3 cells have little capacity to adhere to collagen IV. In contrast, the adhesion of 3T3 cells to collagen IV is altered markedly by the expression of α_1 on their surfaces. The expression of the mutant and wild-type human α_1 subunits results in a functional receptor that confers the ability to adhere to collagen IV. The adherence of all three cell lines to collagen IV is similar, suggesting that the mutations do not affect ligand binding and postligand events. This adhesive capacity is blocked by addition of the anti- α_1 antibody 1B3 (Figure 2D) and by replacement of Mg²⁺ with Ca²⁺ (Figure 2B), characteristic of α_1 -dependent adhesion. Thus the mutation of the membrane proximal residues does not affect the $\alpha_1\beta_1$ -dependent adhesion to collagen IV.

Cellular Localization of Integrin Receptors

To study the cellular localization of integrins in HA1 K>D and HA1 K>A cells, we plated the cells on collagen IV after treatment with cycloheximide to block endogenous matrix production. For both cell lines, when plated on collagen IV, the human α_1 subunit (Figure 3, A and C) localizes to focal contact sites, as shown by colocalization with the cytoskeletal protein talin (Figure 3, B and D). This vigorous focal contact formation on collagen IV is consistent with the wild-type level of adhesion and spreading observed for the mutant α_1 subunits. Thus, mutation

Figure 1. Surface expression of human α_1 subunits in NIH-3T3 cells. (A) Extracts of ^{125}I surface-labeled HA1 K>D cells (a), HA1 K>A cells (b), or SW K>D cells (c) were incubated with mouse monoclonal anti-human α_1 antibody TS2/7. Immunoprecipitates were recovered by the use of goat anti-mouse agarose followed by analysis with SDS-PAGE and autoradiography. Similar levels of surface α_1 expression and α/β heterodimer formation are seen for all cell lines. Arrowheads indicate the position of the α_1 subunits; small arrows show the position of the β subunits. Note the increased electrophoretic mobility of the avian β subunit in the SW K>D cells (c). (B) Flow cytometry analysis of NIH-3T3 cells. NIH-3T3 cells (dotted line, a–d), HA1 cells (a), HA1 K>D cells (b), HA1 K>A cells (c), or SW K>D cells (d) were incubated with the human α_1 -specific monoclonal antibody (mAb) TS2/7 in the presence of 0.1% NaN_3 , and the bound mouse IgG was visualized by FITC-conjugated goat anti-mouse antibody. All four cell lines showed similar expression levels of the human α_1 cDNA.

of the membrane proximal region has no apparent effect on one main postligand event, ligand-dependent focal contact formation.

We have also tested the effect of this mutation on constitutive or ligand-independent focal contact localization. Previously, we have shown that the deletion of the distal α_1 cytoplasmic domain leads to a receptor that localizes to focal contacts even on substrates (such

as FN) to which it does not bind. Therefore, we plated HA1 K>D (Figure 4, A and B) and HA1 K>A cells (Figure 4, C and D) on FN and performed colocalization studies for α_1 integrin subunits, with talin as a marker of focal contact sites. We have shown previously that the wild-type human α_1 subunit is not localized to focal contact sites when plated on FN (Briesewitz *et al.*, 1993b).

Figure 2. Adhesion assays of 3T3 cell lines. NIH-3T3 cells (circles), HA1 cells (squares), HA1 K>A cells (diamonds), or HA1 K>D cells (triangles) were assayed for adhesion to collagen IV or FN. Amounts of adhesive proteins indicated at the bottom of the graphs were dried down on microtiter plates. Adhesion was measured as described (see MATERIALS AND METHODS). Col IV adhesion (A and B) in DMEM medium (A), HEPES-buffered medium containing 1 mM Ca^{2+} (open symbols) or 1 mM Mg^{2+} (filled symbols) (B), and FN adhesion in the presence of Ca^{2+} (open) or Mg^{2+} (filled; C) showed differences in divalent cation requirement for col IV or FN adhesion. Inhibition studies are shown in D. Microtiter well surfaces were coated with FN (0.25 $\mu\text{g}/\text{well}$, open bars) or col IV (0.01 $\mu\text{g}/\text{well}$, black bars). HA1 cells or HA1 K>D cells were incubated for 30 min at 37°C in the presence or absence of the inhibitory anti- α_1 mAb 1B3.1. Adhesion was measured as described (see MATERIALS AND METHODS). Col IV adhesion is blocked by the mAb, whereas FN adhesion is not affected.

In contrast, when the HA1 K>D cells were plated on FN, the human α_1 subunit (Figure 4A) was localized to focal contact sites, as seen by the colocalization with the cytoskeletal protein talin (Figure 4B). Antibody inhibition experiments (Figure 2D) have shown that the K>D mutation does not confer FN binding affinity to the mutant $\alpha_1\beta_1$. In addition, the adhesion to FN is unaffected by addition of Ca^{2+} , in contrast to collagen IV adhesion (Figure 2, B and C). Therefore, the mutant $\alpha_1\beta_1$ localizes to focal contacts in a ligand-independent manner. In contrast, despite the ability of HA1 K>A to form focal contacts on collagen IV, this mutant α_1 subunit (Figure 4C) does not localize to focal adhesions on FN (Figure 4D). Thus, the acquisition of

the ability to localize to focal adhesions on FN by mutant α_1 apparently requires the negatively charged aspartate residue, because the alanine substitution did not lead to the effect.

Analysis of Integrin Receptors with Exchanged Intracellular Domains

The effect of the aspartate substitution at the juxtamembrane region could be either a local effect of the charge or an effect involving distant sequences within the α subunit for the loss of this latency. To investigate the mechanism of this effect, we decided to test the K>D mutation in integrin receptors with swapped

Figure 3. Double-label immunofluorescence of cells expressing mutant α_1 subunits on collagen IV. The HA1 K>D cells (A and B) and HA1 K>A cells (C and D) were plated on collagen IV and stained with a mixture of rabbit anti-talin and mouse monoclonal anti-human α_1 TS2/7. The primary antibodies were visualized by the use of fluorescein-conjugated rabbit anti-mouse IgG (A and C) and rhodamine-conjugated goat anti-rabbit IgG (B and D).

domains. We have shown previously that an exchange of the intracellular and extracellular domains of the α_1 and avian β_1 subunits results in a receptor that does not localize to focal adhesions in the presence or absence of ligand (Briesewitz *et al.*, 1995). Moreover, we showed that removal of the distal α cytoplasmic domains in this context led to ligand-independent focal contact localization in a receptor that was unlikely to transmit outside-in information. If the K>D mutation were causing a relative shift in the relationship of α and β cytoplasmic domains, leading to ligand-independent focal contact localization, then this mutation in the swapped receptors might not be able to cause such a shift. Therefore, $\beta x \alpha c$ K>D was transfected into a cell line expressing $\alpha x \beta c$, and the chimeric receptor $\alpha x \beta c / \beta x \alpha c$ K>D was produced in the resulting cell line. This receptor selectively dimerized and was expressed efficiently on the cell surface at similar levels as the α_1 mutants (Figure 1). However, when these cells were placed on FN, the swapped receptor did not localize to focal adhesions (Figure 5C), which again were identified via talin staining (Figure 5D). These

results show that the mechanism of the loss of latency via the K>D mutation and the deletion of the distal α cytoplasmic domain are different.

Assay of Dominant Negative Phenotype

We have used the β_1 cytoplasmic domain connected to the transmembrane and extracellular domains of the interleukin-2 receptor α subunit (IL2R- β_1) in stably transfected NIH-3T3 cell lines and showed that the addition of LPA leads to a rapid detachment from the substratum, which is dependent on an active cytoskeleton (Smilenov *et al.*, 1994). The IL2R- β_1 construct behaves as a dominant negative, because it effectively engages the cytoskeleton via its active cytoplasmic domain but does not bind to the matrix, leading to detachment. Because the K>D mutant used in this study localizes to focal adhesions on FN, a ligand to which it does not bind, we decided to assay integrin constructs that localize to focal adhesions constitutively for their ability to mediate the LPA-dependent detachment. Therefore, we plated HA1 K>D cells and

Figure 4. Double-label immunofluorescence of cells expressing mutant α_1 subunits on FN. HA1 K>D cells (A and B) and HA1 K>A cells (C and D) were plated on FN and stained with a mixture of rabbit anti-talin and mouse monoclonal anti-human α_1 TS2/7. The primary antibodies were visualized by the use of fluorescein-conjugated rabbit anti-mouse IgG (A and C) and rhodamine-conjugated goat anti-rabbit IgG (B and D).

IL2R- β_1 cells on FN-coated coverslips and compared the effect of LPA treatment (Figure 6). As expected, after 2 min of treatment with LPA, the IL2R- β_1 -containing cells had completely rounded up on the substratum (Figure 6D). Strikingly, the K>D cells did not round up on LPA treatment (Figure 6B) and essentially behaved as wild-type cells. In addition, we have repeated this experiment with the CYT cell line, which contains a deletion of the α_1 cytoplasmic domain distal to the GFFKR sequence. This mutant integrin exhibits ligand-independent focal contact localization (Briesewitz *et al.*, 1993b); however, these cells do not detach from FN upon LPA treatment (Figure 6F). These results suggest that, although both the cytoplasmic domain-deleted form and the K>D mutant of $\alpha_1\beta_1$ integrin are able to localize to focal contacts on FN, they cannot mediate a dominant negative phenotype.

DISCUSSION

Integrins, like most receptors, are latent until they have bound their ligand. Previous investigations

showed that deletion of the α cytoplasmic domain ablated latency and allowed for constitutive downstream effects in the absence of ligand (Briesewitz *et al.*, 1993b; Ylännä *et al.*, 1993; Kawaguchi *et al.*, 1994). In this study, we found that mutating the lysine at the membrane-cytoplasm interface to an aspartic acid caused a similar loss of latency. This mutation gives insight into the structural changes that may be occurring in native integrins when they bind ligand.

Biophysical studies have shown that juxtamembrane amino acids are significantly influenced by the membrane's negative charge. An analysis of the juxtamembrane glutamic acid in the activated (V664E) neu receptor tyrosine kinase revealed that its pKa was drastically increased over its value in solution (Smith *et al.* 1996). Whereas this residue in solution would have a pKa ~ 4.5 and therefore be negatively charged at pH 7.0, its pKa was found to be 9.0 in the vicinity of a negatively charged membrane. At neutral pH, then, the glutamic acid was uncharged and capable of partitioning into the membrane and participating in hydrogen bonds that stabilized dimers.

Figure 5. Double-label immunofluorescence of cells expressing mutant α_1 subunits on FN. HA1 K>D cells (A and B) and SW1 K>D cells (C and D) were plated on FN and stained with a mixture of rabbit anti-talin and mouse monoclonal anti-human α_1 TS2/7. The primary antibodies were visualized by the use of fluorescein-conjugated rabbit anti-mouse IgG (A and C) and rhodamine-conjugated goat anti-rabbit IgG (B and D).

By analogy, the K>D substitution we engineered in the α juxtamembrane domain may partially mimic ligand-induced activation of the integrin. The aspartate can be expected to be protonated in the vicinity of the membrane because of its raised pKa. Now neutrally charged, the aspartate can translocate into the bilayer and/or participate in hydrogen bonding with other residues.

If sliding of the α -chain into the bilayer were solely responsible for the loss of latency, then replacement of the lysine with an alanine also should have mediated the effect, which it did not. We suggest that the increased hydrogen-bonding ability of the juxtamembrane aspartate residue in K>D contributes to the loss of latency, and the alanine in K>A, which can still partition into the membrane, cannot mediate this additional hydrogen bond formation and therefore cannot disrupt latency. This additional bonding could result in a subtle shift in the rotational orientation of the two chains, which causes the integrin's partial activation.

It is possible that, despite its protonation and neutral charge, the aspartate in K>D does not actually cause translocation of the entire polypeptide chain and, instead, partitions into the membrane by effecting a local deformation of the chain's secondary structure. If this were true, then the mutation should function in both native integrins and those with swapped extracellular and intracellular domains, similar to the truncation of the α cytoplasmic domain. Because the K>D mutation did not disrupt latency in swapped integrin heterodimers, we conclude that its effect requires the transmembrane integrity of the two chains and may therefore involve translocation of the entire α subunit.

Does the K>D phenotype reflect the *in vivo* conformational change upon ligand binding? Several lines of evidence suggest that a change in the relative orientations of the two chains is required for integrin function. Unlike growth factor receptors, which probably only require ligand-induced dimerization for function, only a subset of downstream events is induced by antibody-mediated integrin clustering, suggesting

Figure 6. Effect of LPA on NIH-3T3 cell lines. HA1 K>D cells (A and B), 3T3 IL2R- β_1 cells (C and D), and CYT cells (E and F) were plated on FN-coated coverslips and incubated in complete media at 37°C overnight. LPA (5 $\mu\text{g}/\text{ml}$) was added, and cells were photographed immediately (A, C, and E) or after five minutes (B, D, and F) at room temperature. The HA1 K>D and CYT cells remained attached throughout, whereas the 3T3 IL2R- β_1 cells had detached almost completely by 5 min, demonstrating a lack of a dominant negative phenotype in the HA1 K>D and CYT cells.

that additional conformational changes are required (Miyamoto *et al.*, 1995). Furthermore, in ligand-dependent systems, virtually all integrin-dependent phosphorylations are cytochalasin sensitive (Huang *et al.*, 1993), suggesting that cytoskeletal engagement is required for downstream signaling. The failure of the swapped integrin receptors to elicit post-ligand binding events shows that ligand binding cannot activate integrins when transmembrane conformational changes are prevented (Briesewitz *et al.*, 1995). We

postulate that ligand binding causes a relative translocation and rotation of the α cytoplasmic domain leading to FAK phosphorylation and cytoskeletal association, which seem to be mediated by the β cytoplasmic domain. This model is similar to that proposed for inside-out integrin activation (Williams *et al.*, 1994). Recent data suggest that a potential salt bridge, which presumably would prevent conformational changes, could be required for latency (Hughes *et al.*, 1996).

The results presented here and in our earlier work illustrate the relationship between the localization of integrin constructs to preformed focal contacts and their ability to mediate LPA-dependent detachment and allow us to hypothesize on the various functions of the β cytoplasmic domain. Both β cytoplasmic domain reporter constructs and heterodimers with mutated or deleted α cytoplasmic domains are able to localize to focal adhesions constitutively. The latter, however, do not fully engage the actin cytoskeleton, as demonstrated by the cells' lack of LPA-dependent detachment. Although it is possible that we have not achieved a sufficient level of expression in the K>D lines, this explanation is unlikely, because cell lines that exhibit a variety of mutant heterodimer expression levels, including much greater than endogenous levels, do not detach upon LPA treatment, whereas those with even modest levels of single subunit β cytoplasmic domain chimeras do (Figure 6). Instead, we propose that the activities for focal contact localization and actin engagement are separable, presumably attributable to distinct regions of the β cytoplasmic domain. In α cytoplasmic domain truncation mutants and the K>D mutants, the activity within the β cytoplasmic domain necessary for actin interaction is masked by portions of the α -chain. This hypothesis predicts it should be possible to create β cytoplasmic domain mutants in single subunit chimeras that allow them to localize constitutively to focal contacts but do not support LPA-dependent detachment. We are hopeful that such mutants will enable us to understand the regions of the β cytoplasmic domain that link integrins to the actin cytoskeleton.

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