Distinct Functions of Integrin α and β Subunit Cytoplasmic Domains in Cell Spreading and Formation of Focal Adhesions

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Abstract. Integrin-mediated cell adhesion often results in cell spreading and the formation of focal adhesions. We exploited the capacity of recombinant human αtmβ3 integrin to endow heterologous cells with the ability to adhere and spread on fibrinogen to study the role of integrin cytoplasmic domains in initiation of cell spreading and focal adhesions. The same constructs were also used to analyze the role of the cytoplasmic domains in maintenance of the fidelity of the integrin repertoire at focal adhesions. Truncation mutants of the cytoplasmic domain of αtm did not interfere with the ability of αtmβ3 to initiate cell spreading and form focal adhesions. Nevertheless, deletion of the αtm cytoplasmic domain allowed indiscriminate recruitment of αtmβ3 to focal adhesions formed by other integrins. Truncation of the β1 subunit cytoplasmic domain abolished cell spreading mediated by αtmβ3 and also abrogated recruitment of αtmβ3 to focal adhesions. This truncation also dramatically impaired the ability of αtmβ3 to mediate the contraction of fibrin gels. In contrast, the β3 subunit cytoplasmic truncation did not reduce the fibrinogen binding affinity of αtmβ3. Thus, the integrin β3 subunit cytoplasmic domain is necessary and sufficient for initiation of cell spreading and focal adhesion formation. Further, the β3 cytoplasmic domain is required for the transmission of intracellular contractile forces to fibrin gels. The α subunit cytoplasmic domain maintains the fidelity of recruitment of the integrins to focal adhesions and thus regulates their repertoire of integrins.

Cell adhesion is controlled by binding affinity and kinetics of interaction between adhesive ligands and cell surface receptors. In addition, events such as lateral diffusion of receptors (7) and interactions with and reorganization of the cytoskeleton strengthen adhesion (16, 29). Cell adhesion induces changes of cell shape and cytoskeletal organization and regulates cell growth and patterns of gene expression (24, 48, 53). Integrins are transmembrane heterodimeric glycoprotein adhesion receptors present in almost all cells (23). These receptors mediate all of the consequences of cell adhesion enumerated above. Moreover, after initial cell adhesion, many integrins are concentrated at sites of close approximation between cell and substrate. These sites, termed focal adhesions or focal contacts (4), are also sites of end-on insertion of actin microfilaments into the plasma membrane and at which certain cytoskeletal and signaling molecules concentrate (53). Ligand binding to many integrins triggers focal adhesion formation. In addition to initiating focal adhesions, certain mutant integrins (58) or receptor chimeras containing the β1 cytoplasmic domain (27, 49) are recruited to existing focal adhesions without evident ligand binding. Thus, analysis of the role of integrins in formation of focal adhesions may be divided into initiation and recruitment functions.

Integrin cytoplasmic domains are topographically accessible to intracellular cytoplasmic components. Moreover, the β1 cytoplasmic domain binds to focal adhesion proteins such as talin (22) and α-actinin (41). The β1 cytoplasmic domain is required for localization of recombinant αβ1 to existing focal adhesions (19, 31, 56), but the presence of endogenous β1 precluded analysis of initiation in the published work. Preliminary studies reported that the α5 subunit cytoplasmic domain is not absolutely required for focal adhesion formation (Juliano, R. L., J. S. Bauer, L. J. Kornberg, and J. Varner. 1992. Mol. Biol. Cell. 3[Suppl]:94a).

Integrin αmβ3 (platelet GPIIb–IIIa) is a prototype integrin that mediates cell–cell and cell-substratum interaction (43, 44), signals cell spreading (61) and initiates and enters focal adhesions (64, 65). Moreover this integrin is required for the retraction of fibrin clots (5, 37), wherein intracellular contractile forces are transmitted to extracellular fibrin polymers. αmβ3 offers several advantages in the studies of integrin domains which function in cell adhesion. (a) Certain ligands such as fibrinogen (fg)1 and von Willebrand factor promote adhesion via αmβ3, but not through the majority of other integrins. Thus, in many cell lines, events mediated by transfected αmβ3, such as cell adhesion, spreading, and focal adhesion formation, can be analyzed with minimal confounding effects of endogenous integrins. (b) Well character-

1. Abbreviation used in this paper: fg, fibrinogen.
ized quantitative ligand binding assays for αmβ3 are available (32), again without significant contributions from endogenous integrins. (c) An extensive library of αmβ3 mAbs exists. Antibodies specific for occupied (13,14) or activated (54) conformations of αmβ3 are available and they permit precise in situ analysis of receptor function. Other antibodies which inhibit or enhance (13, 14) receptor function can be used for αmβ3 ligand binding studies.

In the present work, we examined the effects of truncations of αmβ3 cytoplasmic domains on initiation of cell spreading and focal adhesion formation. We found that the β3 subunit cytoplasmic domain is necessary and sufficient for these cytoskeleton-related processes and for recruitment of integrins to existing focal adhesions. Further, the β3 cytoplasmic domain was also required for the contraction of fibrin clots, i.e., for the transmission of contractile events to a model extracellular matrix. In contrast, the α subunit cytoplasmic domain, while not required for initiation, regulates the integrin specificity of recruitment to focal adhesions.

Materials and Methods

Cell Culture

Clonal CHO cell lines transfected with human αm and β3 cDNAs or mutants αmβ3(D119V), αmβ3(D278), and αm(D996)β3 in PCM8(2) vector were constructed and characterized as described (28, 38–40). The αm(D996) was produced by introduction of a TAA stop codon immediately downstream of the conserved GFFKR sequence of the αm cytoplasmic domain in the CD2b plasmid (39). HT 1080 Human fibrosarcoma cells were from the American Type Culture Collection (Rockville, MD). Cells were maintained in DME (Whittaker Bioproducts Inc., Walkertown, NC), supplemented with 10% FCS (Gibco BRL Life Technologies, Inc., Gaithersburg, MD), nonessential amino acids (Sigma Immunochemicals, St. Louis, MO), and penicillin and streptomycin (Sigma Immunochemicals).

1 d after passage, cells were harvested and 107 cells/ml were electroporated (360 V. Capacitance = 960 μF, R4) in the presence of 20 μg DNA using a BTX 600 Electro Cell Manipulator (BTX, Inc., San IMago, CA.). After 48 h, cells were harvested and levels of αmβ3 expression determined by flow cytometry as described below. All results described herein were observed on at least three different transfections for each αmβ3 pair. In addition, stable CHO cell lines bearing αmβ3, αmβ3(D119V), αmβ3(D278), and αm(D996)β3 were also used to further confirm results with these recombinant integrins.

Antibodies

Mouse mAb 2G12, which binds to αmβ3 (63) was from Dr. Virgil A. Woods (University of California, San Diego, CA). Rat mAb against α3 integrin subunit Ab 16 (I) was from Dr. Kenneth M. Yamada (National Institute of Dental Research, Bethesda, MD) and the mouse anti-human anti-α3β1 complex LM609 (10) from Dr. David A. Cheresh (The Scripps Research Institute). PAC-1 antibody against activated form of αmβ3 was from Dr. Sanford I. Shattil (54). The mAbs 15, 62 and anti-LIBS1 against integrins αmβ3(A996)//β3 were also used to further confirm results with these recombinant integrins.

Microscopy and Flow Cytometry

Cell spreading assays and immunofluorescence staining were done on 12-mm circular glass coverslips (No. 1; Fisher Scientific Co., Pittsburgh, PA). The coverslips were coated overnight at 4°C with 20 μg/ml of fibronectin in PBS or with 30 μg/ml type I collagen (Nirai Gelatin Co., Osaka, Japan) in 0.05% acetic acid, followed by blocking with 1% BSA (RIBA grade, Sigma Immunochemicals). The cells were detached with 0.5 μl of trypsin, 0.5 mM EDTA (Irvine Scientific, Santa Ana, CA), washed with DME containing 10% FCS, washed twice with the same medium without serum, and seeded on the coverslips in serum-free medium for indicates times. CHO cells were fixed with 2% parafomaldehyde, 0.5% Triton X-100 for 10 min on ice, and HT1080 cells with methanol for 10 min at −20°C. The number of spread cells was counted under a phase contrast microscope by two independent observers. Flattened cells with regular margin were defined as spread. Data are reported as mean percent of spread cells from four high power microscopic fields (total number of counted cells > 200). In some experiments, cell lines expressing αmβ3 or αmβ3(D278) were allowed to adhere on fibronectin-coated coverslips for 90 min and then 200 μM GRGDSP peptide (Peninsula Laboratories, Inc., Belmont, CA) was added to the media. After different time points cells were fixed and stained with anti-αm (FL98DF6). The percentage of cells in which αmβ3 was at focal adhesions was estimated by fluorescence microscopy. At each time point >150 cells were counted. All experiments were repeated at least three times.

For immunofluorescence staining of the fixed cells, coverslips were incubated 30 min with the primary antibody in PBS, washed twice with PBS and incubated for another 30 min with FITC-conjugated goat anti-mouse IgG (ab19) (Tago Inc., Burlingame, CA) FITC-conjugated goat anti-rabbit IgG (Sigma Immunochemicals). For double staining the specimens were treated with irrelevant mouse IgG and then stained with biotin-labeled PL98DF6 followed by TRITC-coupled streptavidin (Molecular Probes, Inc., Eugene, OR). The coverslips were then washed and mounted in FITC-Guard™ mounting media (Testog Inc., Chicago, IL). The specimens were examined with a Leitz Orthoplan microscope with plan Apochromat 100x oil immersion objective and photographs were taken on Kodak Tmax 400 film (Eastman Kodak Co., Rochester, NY). As controls, specimens were stained with irrelevant antibodies or primary antibody was omitted.

For flow cytometry analysis of αmβ3 expression or activation status, the cells were stained in suspension for 30 min at 22°C with saturating concentrations of purified mAb 2G12 IgG or PAC-1 ascites. After washing, cells were then incubated with FITC-conjugated anti-mouse IgG or IgM (Tago Inc.) for 30 min, and then washed and stained with biotin-labeled PL98DF6 followed by TRITC-coupled streptavidin (Molecular Probes, Inc., Eugene, OR). The coverslips were then washed and mounted in FITC-Guard™ mounting media (Testog Inc., Chicago, IL). The specimens were examined with a Leitz Orthoplan microscope with plan Apochromat 100x oil immersion objective and photographs were taken on Kodak Tmax 400 film (Eastman Kodak Co., Rochester, NY). As controls, specimens were stained with irrelevant antibodies or primary antibody was omitted.

Fibrin Clot Contraction

These assays were performed by a modification of published methods (30, 36). Briefly, 3 × 107 cells in 350 μl of DME containing 25 mM Hepes were added to 10 × 75 mm glass tubes (Fisher Scientific Co.). 200 μl of pooled human platelet fibrinogen-depleted plasma anticoagulated with 0.38% Na Citrate was added followed by 200 μl of Hepes-DME containing 28 mM CaCl2 and 5 μM human thrombin (Sigma Immunochemicals). The tubes were subsequently incubated at 37°C for 2 h and clot retraction was estimated visually.
Figure 1. (A) Adhesion of CHO cells to \( \text{fg} \). Cells were allowed to adhere for 30 min at 37°C and adhesion was quantified as described in Materials and Methods. Stable \( \alpha_{\text{in}}\beta_3 \)-bearing CHO cell lines (○) adhered readily, but parental CHO cells (△) or cells bearing \( \text{fg} \)-binding deficient mutant \( \alpha_{\text{in}}\beta_3(D119-Y) \) (□) failed to adhere. (B) Expression of wild-type and mutant \( \alpha_{\text{in}}\beta_3 \) in CHO cells. On the same day of the adhesion experiment performed in A, cells bearing \( \alpha_{\text{in}}\beta_3 \) (fine solid line) or \( \alpha_{\text{in}}\beta_3(D119-Y) \) (dashed line) were stained with an anti-\( \alpha_{\text{in}}\beta_3 \) (2G12). Surface expression levels were estimated by flow cytometry. Both recombinant \( \alpha_{\text{in}}\beta_3 \)s were expressed at similar levels. CHO cells were negative (bold solid line).

Results

The \( \alpha \) Subunit Cytoplasmic Domain Is Not Required for Initiation of Spreading and Focal Adhesion Formation

To study the role of the cytoplasmic domains in integrin function, we used CHO cells transfected with human \( \alpha_{\text{in}}\beta_3 \) integrin. Transfection of \( \alpha_{\text{in}}\beta_3 \) resulted in de novo acquisition of adhesion to \( \text{fg} \) (Fig. 1 A). Moreover, this adherence was dependent on the expression of functional \( \alpha_{\text{in}}\beta_3 \), since \( \alpha_{\text{in}}\beta_3(D119-Y) \), which lacks \( \text{fg} \) binding function (28), failed to mediate adhesion (Fig. 1 A), even though it was expressed at the same level as \( \alpha_{\text{in}}\beta_3 \) (Fig. 1 B). Furthermore, the \( \alpha_{\text{in}}\beta_3 \)-bearing cells were able to spread on \( \text{fg} \), and \( \alpha_{\text{in}}\beta_3 \) was localized at focal adhesions (Fig. 2 a). The identity of these focal adhesions was confirmed by the presence of talin (Fig. 2 b). Thus, CHO cell adhesion, spreading, and focal adhesion formation on \( \text{fg} \) was dependent on the transfected integrin.

Figure 2. Identification of \( \alpha_{\text{in}}\beta_3 \) and \( \alpha_{\text{in}}(\Delta996)\beta_3 \) in focal adhesions. Stably transfected cells were permitted to adhere to \( \text{fg} \) for 2 h at 37°C. Double label immunofluorescence staining of \( \alpha_{\text{in}}\beta_3 \) (a and b) and \( \alpha_{\text{in}}(\Delta996)\beta_3 \)-bearing cells (c and d) with biotinylated PL98DF6 (anti-\( \alpha_{\text{in}} \)) (a and c) and polyclonal anti-talin antibodies (b and d) is depicted. Note that both the cells are well spread and \( \alpha_{\text{in}} \) reactivity is found at focal adhesions identified by talin immunoreactivity. In control staining with irrelevant biotinylated antibodies, no colocalization was detected (not shown). Bar, 10 \( \mu \text{m} \).
Cells bearing the α subunit truncation mutant α\textsubscript{m}(Δ996)β\textsubscript{3}, spread on \textit{fg} and the truncated α subunit was localized at focal adhesions (Fig. 2, c and d). This suggests that the bulk of the α\textsubscript{m} cytoplasmic domain is not required for initiation of spreading and focal adhesion formation. CHO cells express low levels of endogenous α\textsubscript{m}, which might complex with transfected β\textsubscript{3}. To rule out any contribution of α(hamster)β(human) heterodimers to initiation of spreading and focal adhesions, we exploited the reactivity of mAb LM609 with such heterodimers. Cells transfected with only human β\textsubscript{3} reacted with mAb LM609 (not shown) and acquired the capacity to adhere to \textit{fg}. LM609 completely inhibited the adhesion of cells transfected with β\textsubscript{3} alone, whereas it had no effect on the adhesion of α\textsubscript{m}(Δ996)β\textsubscript{3}-bearing cells (Fig. 3 a). Furthermore, the α\textsubscript{m}(Δ996)β\textsubscript{3}-bearing cells were able to spread and form anti-α\textsubscript{m} reactive focal adhesions in the presence of the mAb LM609 (Fig. 3, b and c). This shows that the α\textsubscript{m}(Δ996)β\textsubscript{3} complex initiates cell adhesion, spreading, and focal adhesions on \textit{fg}. Thus, the bulk of α\textsubscript{m} subunit cytoplasmic tail is not needed for these processes.

**Deletion of the α Subunit Cytoplasmic Domain Causes Indiscriminate Recruitment of α\textsubscript{m}β\textsubscript{3} to Focal Adhesions Formed by Other Integrins**

To study the recruitment of the α\textsubscript{m} truncation mutant to focal contacts we cultured cells bearing it or wild-type α\textsubscript{m} on fibronectin. In contrast to α\textsubscript{m}β\textsubscript{3} (Fig. 4 a), α\textsubscript{m}(Δ996)β\textsubscript{3} (Fig. 4 c) was localized at focal adhesions on fibronectin. β\textsubscript{3} (Fig. 4 d) and α\textsubscript{3} (not shown) immunoreactivities colocalized with that of α\textsubscript{m}(Δ996). This organization was not dependent on α\textsubscript{m}(Δ996)β\textsubscript{3} binding to fibronectin, since even when expressed together with a ligand-binding deficient β\textsubscript{3} mutant, β\textsubscript{3}(D119→Y) (28), α\textsubscript{m}(Δ996) was found at focal adhesions in cells cultured on fibronectin (Fig. 4 e). A similar pattern was observed when the cells were stained for β\textsubscript{3} (not shown). Since cotransfection of β\textsubscript{3} subunit is required for α\textsubscript{m} surface expression (38), β\textsubscript{3} was the only β subunit associated with α\textsubscript{m}(Δ996). Thus, the staining of focal adhesions for both α\textsubscript{m} and β\textsubscript{3} shows that α\textsubscript{m}(Δ996)β\textsubscript{3}(D119→Y) could be recruited to focal adhesions formed by αβ\textsubscript{3} in spite of a profound defect in ligand binding.

Since recruitment to focal adhesions is a feature of ligand-bound integrins (27), we compared the reactivity of α\textsubscript{m}β\textsubscript{3} and α\textsubscript{m}(Δ996)β\textsubscript{3} with mAbs specific for the ligand-occupied or activated forms of α\textsubscript{m}β\textsubscript{3}. Either with anti-LIBS1 (Fig. 5 A) or PAC-1 (Fig. 5 B) antibodies, only a low basal reactivity was detected. The anti-LIBS1 and PAC-1 binding was dramatically increased by the appropriate inducing agents (RGDS peptide or activating antibody, respectively). Thus, in contrast to the previously characterized α\textsubscript{m} subunit cytoplasmic mutations (40), the α\textsubscript{m}(Δ996) truncation did not result in detectable conformational changes of the extracellular domain of the receptor.

To determine whether recruitment of α\textsubscript{m}(Δ996) to focal adhesions is specific for CHO cells, for fibronectin substrata, or for focal adhesions formed by αβ\textsubscript{3}, we transiently transfected α\textsubscript{m}β\textsubscript{3} or α\textsubscript{m}(Δ996)β\textsubscript{3} into 1T1108 human fibrosarcoma cells. These cells express αβ\textsubscript{3} and αβ\textsubscript{1} and spread on both fibronectin and collagen. On fibronectin, αβ\textsubscript{3} was detected at focal adhesions (Fig. 6 g) and on collagen αβ\textsubscript{3} (Fig. 6 f) was in the focal adhesions. On both of these substrates, α\textsubscript{m}(Δ996) (Fig. 6, a and b) but not wild-type α\textsubscript{m}β\textsubscript{3} (Fig. 6 c) completely inhibited the adhesion of cells transfected with wild-type αβ\textsubscript{3} (Fig. 6 d). This shows that the α\textsubscript{m}(Δ996)β\textsubscript{3} complex initiates cell adhesion, spreading, and focal adhesions on fibronectin. Thus, the bulk of α\textsubscript{m} subunit cytoplasmic tail is not needed for these processes.

![Figure 3](image-url)
Figure 4. Ligand-binding independent recruitment of $\alpha_{\mathrm{m}(\Delta 996)}\beta_3$ to focal adhesions. CHO cells were transfected and after 48 h were cultured on fibronectin substrates for 2 h. The cells were then stained with biotinylated anti-human $\alpha_{\mathrm{m}}$ (mAb PL98DF6) (a, c, e, and f) or anti-hamster $\beta_3$ (7E2) (b and d). The same cell was photographed in a, b, and c, d. The transfected integrins were $\alpha_{\mathrm{m}}\beta_3$ (a and b), $\alpha_{\mathrm{m}(\Delta 996)}\beta_3$ (c and d), $\alpha_{\mathrm{m}(\Delta 996)}\beta_3(D119\rightarrow Y)$ (e), and $\alpha_{\mathrm{m}}\beta_3(D119\rightarrow Y)$ (f). $\alpha_{\mathrm{m}(\Delta 996)}$ (c), but not wild-type $\alpha_{\mathrm{m}}$ (a), was detected at focal adhesions containing hamster $\beta_3$. Ligand binding defective $\alpha_{\mathrm{m}(\Delta 996)}\beta_3(D119\rightarrow Y)$ was detected at focal adhesions in cells cultured on fibronectin (e), while $\alpha_{\mathrm{m}}\beta_3(D119\rightarrow Y)$ had a uniform cell surface distribution (f).
Lack of spontaneous anti-LIBS1 binding to αmβ3. CHO cells bearing αm(Δ996)β3 or αmβ3 were incubated with 275 nM 125I-anti-LIBS1 in the presence (m) or absence (a) of 1 mM RGDS peptide. Anti-LIBS1 binding was estimated as described in Materials and Methods and results were expressed as molecules bound/cell ± SE of triplicates. (B) αm(Δ996)β3 is in a low affinity state. 50 μl of a suspension of 4 x 10^6 αm(Δ996)β3-bearing CHO cells/ml were incubated with fluorescein-conjugated PAC1 in the presence (dotted line) or absence (solid line) of 1 mM RGDS peptide. In the right panel, the cells were stimulated with 2 μM mAb 62. After 20 min at room temperature, the suspension was diluted with 450 μl Tyrode's buffer and PAC binding was measured by flow cytometry as described (39).

Integrin β3 Subunit Cytoplasmic Domain Is Necessary for Cell Spreading and Initiation of Focal Adhesions but not for Ligand Binding

Only 0.6 ± 0.4% of cells expressing αmβ3 with a β3 subunit truncation mutant β3(Δ728), spread within one h of plating on fg. In contrast, 83 ± 1.4% of cells bearing wild-type αmβ3 were spread. To find out whether β3(Δ728)-bearing cells failed to spread on fg because of defects on ligand binding or because of defects in post-occuency events in cell adhesion, we measured the capacity of the αmβ3(Δ728)-bearing cells to bind soluble fg and to adhere to fg-coated surfaces. After activation with mAb62 (39), αmβ3(Δ728)-bearing cells bound fg with an affinity constant, Ka, of 7.92±10^6 ± 0.96×10^6 M^-1 (Kd = 126 nM). This was not significantly different from the affinity constant for αmβ3-bearing cells (9.1±10^6 ± 1.5×10^6 M^-1, Kd = 110 nM) (39). Thus, the cytoplasmic truncation of β3 did not alter the affinity of activated αmβ3 for soluble fg.

In short-term adhesion assays, when either the αmβ3 or αmβ3(Δ728)-bearing cells were not spread (Fig. 7, b and d), both cells adhered equally well on fg (Fig. 7 a, 20 min). After a 1-h incubation at 37°C, when the αmβ3-bearing cells were spread (Fig. 7 e), they adhered better (Fig. 7 a, 1 h) than αmβ3(Δ728)-bearing cells which were not spread (Fig. 7 c). Together with the data that the β3 subunit truncation does not affect the fg binding affinity of αmβ3, this shows that its initial interaction with fg is similar to the wild-type receptor. In contrast, because of the inability of αmβ3(Δ728) to provoke post-occupancy events, the long-term adhesion mediated by αmβ3(Δ728) is less stable than that mediated by wild-type αmβ3.

The β3 Cytoplasmic Domain Is Required for Recruitment to Existing Focal Adhesions

To study the recruitment of αmβ3 and αmβ3(Δ728) to existing focal adhesions, we chose CHO cells expressing these complexes to spread on fibronectin and form complexes containing αβ3. The redistribution of αmβ3 to these focal adhesions was then analyzed by exploiting the findings of LaFlamme and co-workers (27) that soluble ligand binding promotes integrin recruitment to focal adhesions. As described above, wild-type αmβ3 was excluded from the focal adhesions formed under these conditions. After addition of GRGDSP peptide, αmβ3 was localized at focal adhesions in 80% of the cells. In contrast, <10% of cells had αmβ3(Δ728) at focal adhesions before or after peptide addition (Fig. 8). Thus, αmβ3 is recruited to focal adhesions after addition of soluble ligands and this recruitment is dependent on the β3 subunit cytoplasmic domain.

The β3 Cytoplasmic Domain Is Required for Contraction of Fibrin Gels

The foregoing experiments strongly implicate the β3 cytoplasmic domain in linkage of the integrin to cytoskeleton-dependent events. To directly assess the functional significance of this potential linkage, we examined the effect of removal of this domain on the capacity of αmβ3 to mediate fibrin clot contraction. Cells transfected with αmβ3 produced ~50% reduction in the volume of fibrin clots over 2 h. In contrast, untransfected CHO cells failed to retract such clots establishing that this cytoskeleton dependent contractile event was mediated by the recombinant integrin. The β3 truncation mutant also failed to contract the clot (Fig. 9) even though it expressed comparable levels of the recombinant integrin. Thus these data establish that the β3 cytoplasmic domain is required for transmission of contractile force to the fibrin matrix.

Discussion

The major findings of this paper are: (a) the β3 subunit cytoplasmic domain is necessary and sufficient for αmβ3-mediated cell spreading, but its truncation does not change intrinsic fg binding function; (b) the capacity of αmβ3 to
Figure 6. Ligand-independent recruitment of \( \alpha_m(\Delta 996)\beta_3 \) to focal adhesions occurs on multiple substrates, with multiple \( \beta_3 \) integrins and in multiple cell types. HT1080 human fibrosarcoma cells were transiently transfected with either \( \alpha_m(\Delta 996)\beta_3 \) (a and b) or \( \alpha_m\beta_3 \) (c and d). After 48 h, cells were cultured for 2 h on fibronectin (a, c, e, and g) or collagen (b, d, f, and h). Anti-\( \alpha_m \) (PL98DF6) revealed focal adhesions in \( \alpha_m(\Delta 996)\beta_3 \)-bearing cells on both fibronectin (a) and collagen (b). In \( \alpha_m\beta_3 \)-bearing cells no such localization was observed on either substrate (c and d). Anti-\( \alpha_2 \) mAb (R2-8C8, e and f) stained focal adhesions in HT1080 cells cultured on collagen (f) but not on fibronectin (e). Anti-\( \alpha_5 \) mAb (Ab 16; g and h) stained focal adhesions on fibronectin (g), but not on collagen (h).

mediate retraction of fibrin clots is dependent on the \( \beta_3 \) cytoplasmic domain; (c) the bulk of the \( \alpha_m \) subunit cytoplasmic domain is not needed for initiation of cell spreading and focal contact formation by \( \alpha_m\beta_3 \); and (d) \( \alpha_m \) subunit cytoplasmic domain truncation allows ligand independent integrin recruitment to focal adhesions formed by other integrins. Thus, the \( \alpha \) subunit cytoplasmic domain serves to limit integrin recruitment to existing focal adhesions.

The \( \beta_3 \) cytoplasmic domain was both necessary and sufficient for initiation of cell spreading and focal adhesion formation. The failure of \( \alpha_m\beta_3(\Delta 728) \) to provoke these processes establishes necessity. Conversely the capacity of
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**Figure 7.** The β3 cytoplasmic domain is required for initiation of cell spreading. Stable cell lines bearing α_mβ3 or α_mβ3(Δ728) adhere to fn for 20 min at 22°C or 1 h at 37°C. (a) Quantification of adhesion. (b and c) Phase contrast micrographs of α_mβ3(Δ728) cells after 20 min and 1 h, respectively. (d and e) Phase contrast micrographs of α_mβ3 cells after 20 min and 1 h, respectively. α_mβ3(Δ728)-bearing cells were not spread in either conditions. α_mβ3-bearing cells were not spread at 20 min (d) but were spread after 1 h (e).

α_mβ3 that lacked most of the α_m cytoplasmic domain to support these processes establishes sufficiency with respect to integrin cytoplasmic elements. These activities of α_mβ3 may be related to its capacity to stimulate Na^+ -H^+ exchange (3) or activate tyrosine kinases (15). Indeed, the β3 cytoplasmic domain is similar to that of β1 and is involved in both these signaling events (17, 53). In addition, based on similar logic, the β3 cytoplasmic domain was necessary and sufficient for recruitment to existing focal adhesions. The β1 and β3 cytoplasmic domains are interchangeable for localization to focal adhesions (57). Moreover, chimeras containing the cytoplasmic domain of β3 linked to the IL-2 receptor (Tac) (27) or to N-Cadherin (49) are recruited to focal adhesions. Thus, these findings may be applicable to many integrin classes. Studies using purified integrins and synthetic peptides from the cytoplasmic domain of β3 integrin subunit have shown that specific interaction sites for cytoplasmic focal adhesion proteins talin and α-actinin reside in the β3 cytoplasmic domain (22, 41). The binding of β3 subunit cytoplasmic domains to cytoplasmic focal adhesion proteins would provide a cogent mechanism for recruitment. The β1 cytoplasmic sequences involved in localization to focal adhesions have been mapped (19, 31, 47, 56) and at least one site resides within an α-actinin binding motif (47). In addition to α-actinin binding motif, β3 shares motifs with several other β subunit cytoplasmic domains. In particular, there is an NPXY(F) sequence which may form a tight turn (9) and mediate internalization of cell surface receptors. α_mβ3 is internalized and mediates the endocytosis of fn and its storage in secretion granules (18, 62), possibly via the NPXY(F) motif. Moreover, mutations in these motifs inhibited localization of β3 integrins (47) and the adhesive function of a β3 integrin (20). Similarly, the β3 cytoplasmic domain shares the triplet of hydroxylated residues involved in the adhesive function of α_mβ3(20). The fine mapping of the sites in the cytoplasmic domain of β1 involved in initiation of spreading, and comparison with the sites involved in recruitment, signaling, and internalization, should elucidate the hierarchy of these events.

In spite of the role of β3 subunit cytoplasmic domain in cell spreading and focal adhesion formation, there was no...
with high affinity (45) nor mediate cell adhesion to fibronectin (26, 51). Truncation of the $\alpha_{in}$ cytoplasmic domain removed the constraint on entry of $\alpha_{in}\beta_3$ into focal adhesions. The effect of truncation was not due to activation of the ligand binding function of $\alpha_{in}\beta_3$ because: (a) $\alpha_{in}(\Delta 996)\beta_3$ was not activated as judged by PAC1 binding; (b) $\alpha_{in}(\Delta 996)\beta_3$ entered focal adhesions formed on collagen even though $\alpha_{in}\beta_3$ apparently does not bind to collagen (50); and (c) $\alpha_{in}(\Delta 996)\beta_3$, bearing a point mutation which abrogates measurable ligand binding, was recruited to focal adhesions formed on fibronectin. These experiments suggest that the $\alpha$ subunit cytoplasmic domain inhibits interactions of the $\beta$ subunit cytoplasmic domain with cytoskeletal elements and thus constrains recruitment to focal adhesions. Ligand binding to integrins relieves this constraint (27). In support of this, electron microscopic images of purified integrins (6, 35, 60) show that the relationships of the strands containing the transmembrane domains are quite variable and a suggestion that ligand binding might influence this relationship (60). It is possible that there might also be $\alpha$ subunit-specific recruitment of unoccupied integrins to focal adhesions. This is based on the wide (140 nm) spacing required for ligands to initiate focal adhesions (33). At this spacing, unoccupied integrins could readily pack with occupied integrins. Moreover, ligand binding defective $\alpha_{in}\beta_3$ is recruited to focal adhesions formed by wild-type receptor (58). It seems likely that the process of recruitment may be a complex function of integrin interactions on both faces of the plasma membrane as well as potential lateral interactions between integrins.

The present work has centered on focal adhesions and cell spreading, in vitro correlates of integrin-dependent cytoskeletal reorganization. Contraction of extracellular matrix gels is a function of the cytoskeleton relevant to the apposition of wounds and consequent healing (52). This process is dependent on integrins (5, 37, 52) and previous work suggested that the $\alpha$ subunit cytoplasmic domains are involved (8). In particular, recombinant $\alpha_{in}\beta_3$ failed to contract collagen gels when the cytoplasmic domain of $\alpha_1$ was replaced with that of $\alpha_\lambda$. The present work establishes that the $\beta_3$ subunit cytoplasmic domain is essential for the integrin-mediated transmission of contractile force to the extracellular matrix. It seems that ligand binding removes a constraint imposed by the $\alpha$ cytoplasmic domain on cytoskeletal linkage to the $\beta$ subunit. Thus, it is possible that collagen binding to the extracellular domain of chimeric $\alpha_{in}\beta_3$, fails to relieve the constraint imposed by the $\alpha_\lambda$ cytoplasmic domain.

Prior studies (27, 49) and the present work suggest rules governing the integrin repertoire at focal contacts. (a) Integrin $\beta_1$ or $\beta_3$, cytoplasmic domains are necessary and sufficient for initiation of cell spreading and focal adhesion formation. $\alpha$ subunit cytoplasmic domains are not required for initiation of these processes in $\beta_1$ or $\beta_3$ integrins. (b) $\alpha$ subunit cytoplasmic domains limit recruitment by constraining interactions of the $\beta_1$ or $\beta_3$ cytoplasmic domains with components of the focal adhesions. (c) The constraints on recruitment imposed by the $\alpha$ subunit cytoplasmic domain can be eliminated by its deletion or by ligand binding to the integrin.

Integrin cytoplasmic domains influence cellular signaling elements including Tyrosine kinases, phospholipid metabolism, and ion transporters (33). Moreover, different integrins...
may elicit differing responses. Since focal adhesions concentrate a number of signaling molecules, they may be instrumental in integrin signaling. The repertoire of integrins assembled at focal adhesions may then specify the consequences of cell adhesion for growth, migration, and gene expression. Loss of the capacity of a subunit cytoplasmic domains to constrain recruitment through mutation, splicing variants (59), phosphorylation (42, 55), or other posttranslational modification could alter the repertoire of integrins at focal adhesions. This could dramatically alter cellular responses to positional cues provided by the extracellular matrix.

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