Identification of a Domain on the Integran α₅ Subunit Implicated in Cell Spreading and Signaling*

(Received for publication, April 9, 1998, and in revised form, August 6, 1998)

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*This study was supported by National Institutes of Health Grant GM23244. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡‡¶¶ The abbreviations used are: mAb, monoclonal antibody; CHO, Chinese hamster ovary; CMP–HH buffer, Ca²⁺–Mg²⁺–free Hepes-Hanks' buffer; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule.

The α₅β₃ integrin is a cell surface receptor for fibronectin implicated in several cellular activities including cell proliferation, differentiation, and migration. The primary site at which the α₅β₃ integrin interacts with fibronectin is the RGD (Arg-Gly-Asp) amino acid sequence. In general, the sites on the integrin α subunits involved in ligand binding are not well characterized. Based on previous cross-linking studies, sequence alignment, predicted conformation, and intron-exon boundaries, we identified a 144-residue region (positions 223–367) on the α₅ subunit as a putative binding region and divided it into four subdomains named domains I, II, III, and IV. Chimeric receptors were prepared in which sequences on the α₅ subunit were exchanged with the corresponding sequences on the α₅ subunit, which is specific for laminin and does not bind via an RGD sequence. The mutated human α₅ integrin gene was transfected into CHO B2 cells, which are deficient in α₅ expression. Only chimeras of domain III or IV express on the cell surface. Both of these chimeras decreased the adhesion, spreading, focal adhesion assembly, and migration on fibronectin. The adhesion of the chimeric receptors to fibronectin remained sensitive to the RGD peptide, and antibodies that inhibit interaction with the fibronectin synergy site and RGD loop remained inhibitory for the chimeras, indicating that our chimeras do not inhibit binding to either the RGD or synergy sites. Finally, the affinity of soluble fibronectin to cells via the α₅β₃ receptor decreased only about 3-fold. This decrease is substantially less than the observed effects on migration and spreading, which were not altered by changes in substrate concentration. Thus, the alteration in binding sites does not easily account for the changes in cell spreading and focal adhesion assembly. The tyrosine phosphorylation and focal adhesion assembly that are seen when cells expressing the wild type α₅β₃ receptor adhere to fibronectin were inhibited in cells expressing the chimeric receptors. Therefore, our results suggest that the chimeras of these domains likely interrupt α₅-mediated conformational signaling.

Integrins are a large family of heterodimeric adhesive receptors of major importance in diverse biological processes that include embryonic development, tumorigenesis, blood clotting, inflammation, and wound healing (1). Through their dual functions as receptors for molecules associated with the cytoskeleton and for adhesive molecules in the extracellular matrix, on other cells, and in the circulation, they provide anchorage for cell attachment and spreading. Integrins also transduce signals from the outside to the inside of the cells in cooperation with growth factor receptors (2, 3).

The interactions of integrins with their extracellular ligands are subjects of intensive study. The binding affinity is regulated by a process called inside-out signaling, in which the intracellular signals affect affinity and, for some integrins, specificity as well (4). Ligand affinity can also be modulated by exogenous agents, which include the nature of the cations that bind to the integrin; antibodies that induce high affinity ligand binding; and alterations in the membrane proximal region of integrin cytoplasmic domains (5, 6). These observations have been incorporated into the hinge model of integrin function. In this model, affinity/specificity-determining conformational changes are propagated from the inside to the outside via a critical hinge region, which resides near the membrane on the cytoplasmic domains of the integrin subunits (4, 7, 8).

Due to their potential for the design of therapeutics, the ligand-binding sites on integrins are under intensive study. Peptide cross-linking analyses of mutant integrins, peptides corresponding to integrin sequences, and epitope mapping of inhibitory mAbs have been used to identify the integrin ligand-binding sites. From these studies, three regions are implicated in ligand binding: the N-terminal portions of both the α and β subunit and the A (or I) domain in the integrins that contain it (9).

The ligand-binding sites on the β₃ and β₅ subunits are the best understood. RGD-containing peptides cross-link to the N-terminal portion of the β₃ (positions 109–171) subunit (10). The importance of this region in ligand binding of β₃ and some other integrins has been confirmed in several studies. A labeled RGD peptide cross-links to a peptide corresponding to the amino acids 61–203 on the sequence of the β₃ subunit (11). A naturally occurring mutation on β₃ (Asp→Tyr) and mutations of the homologous residue on β₃ and β₅ also result in reduced ligand binding (12, 13). In addition, site-directed mutagenesis within this domain inhibits ligand binding (14).

For integrin α subunits that do not possess an A domain, ligand binding localizes to the N-terminal portion. This region corresponds roughly to the amino-terminal 400 amino acids on several α subunits including α₄, α₅, and α₆b (9). It contains metal ion binding sites and is composed of seven homologous, repeated domains, recently termed FG-GAP repeats (15). Pep-
The importance of divalent metal-binding sites is also seen in the cation dependence of integrin ligand binding. Mn$^{2+}$ and Mg$^{2+}$ generally elevate ligand binding, whereas Ca$^{2+}$ inhibits it (22). Alterations in some EF-hand motifs of the $\alpha$ subunit inhibit ligand binding. The missing 12th coordination site on the partial EF-hand motif on $\alpha$ subunits has led to the hypothesis that integrin ligands may directly bind to cations by providing an oxygenated residue, e.g., the Asp in the RGD sequence (23). Another model suggests that divalent cations promote ligand binding by switching the integrin into an active conformation (6, 24). In most integrins, the binding of Mn$^{2+}$ increases the binding affinity. In addition, changing any one of several different cation-binding sites on $\alpha$ reduces ligand binding similarly, suggesting a structural rather than a direct ligand binding role (19). Although it is not clear which model reflects the real function of cations in integrin-ligand interaction, cation binding is very likely a key factor in this process.

Despite the characterization of cation binding as critical to ligand binding, ligand-binding motifs on integrin $\alpha$ subunits (outside of the I domain on some $\alpha$ subunits) are not well characterized. This is due, in part, to the sensitivity of $\alpha$ subunit to conformational changes that result in improper folding and/or impaired dimerization. In an attempt to avoid this problem, we constructed chimeric integrins to study the ligand binding domain on the $\alpha$ integrin. We selected four consecutive small fragments near the first and second cation-binding site on the $\alpha$ subunit, exchanged each segment with the homologous sequence on the $\alpha$ subunit, and expressed the resulting chimeras in CHO B2 cells, which are deficient in expression of the $\alpha$ subunit (25). We chose $\alpha_{5}$ and $\alpha_{v}$ integrins because both dimerize with the $\beta_{3}$ subunit but have distinct ligand specificity; the former binds to fibronectin, while the later binds to laminin. We found that two of the chimeras express stably on the cell surface and exhibit defects in adhesion, spreading, and migration on fibronectin. Interestingly, the binding to fibronectin via the RGD and synergy sites was not grossly perturbed. However, the mutations did impair focal adhesion formation and adhesion-mediated protein tyrosine phosphorylation. The data suggest that the regions of these mutations are probably involved in outside-in signal transduction.

**EXPERIMENTAL PROCEDURES**

Construction of Wild Type and Chimeric $\alpha$ Subunits—The $\alpha_{5}$ cDNA was originally obtained in the pB Apr-1-neo vector (26) and subcloned into the eucaryotic expression vector, pSV Neo (27). Five restriction sites, BglII, SacII, NheI, BssHII, and NalI, were introduced at the borders of the swapped regions by site-directed mutagenesis as described in the Mutagen-Enzyme$^{TM}$ in vitro mutagenesis kit (Bio-Rad). The $\alpha_{5}$ cDNA bearing the generated restriction sites was cloned into the pTZ-18R vector, and the fragments of interest were exchanged between $\alpha_{5}$ and $\alpha_{v}$ by restriction enzyme digestion and ligation. The chimeric cDNAs were cloned into the expression vector pSV Neo to produce the chimeric integrins. To construct the chimeras with cytoplasmic domain truncations, stop codon sequence was introduced after the GFKKR sequence by site-directed mutagenesis. The chimeric cDNAs were also cloned into pRSVneo.

Transfection and Selection of $\alpha$-Expressing Cells—CHO B2 cells were grown in minimal essential medium (Life Technologies, Inc.) with 10% fetal bovine serum (full medium). 3 × 10$^5$ cells were plated onto 60-mm tissue culture plates for 16–20 h. 8 µg of plasmid DNA and 25 µl of lipofectamine (Life Technologies) were mixed and diluted in minimal essential medium to a final volume of 250 µl. The mixture was incubated for 45 min before addition to the cell cultures. Cells were incubated for 5 h, and the DNA-lipofectamine mixture was replaced by full medium. After 24 h, the cells were moved to selection medium containing 1 mg/ml G418 (Life Technologies), see ing 1% of $\alpha_{5}$, and then maintained in 0.2 mg/ml G418. The expression of $\alpha_{5}$ on the cell surface was analyzed by flow cytometry. The cells were first incubated for 30 min with 20 µg/ml VIF4 antibody in Ca$^{2+}$-, Mg$^{2+}$-free Heps-Hanks’ buffer (CMF-HH buffer) containing 2% BSA and then detached from the plates using 0.2 g/ml EDTA in CMF-HH buffer. The cells were then washed with CMF-HH buffer and resuspended in the same buffer. The cell sorting was performed on an EPICS$^{TM}$-C (Coulter Electronics, Inc., Miami Lakes, FL) flow cytometer equipped with Cisero software for data analysis. Untransfected cells were used as controls. The transfected cells expressing 20-fold over that of the untransfected cells were sorted and enriched.

Adhesion Assays—96-well, non-tissue culture plates were coated with fibronectin in phosphate-buffered saline (PBS) at the indicated concentration for 2 h at 37 °C. The wells were then washed with PBS and blocked with 2% BSA in PBS for 1 h. Cells were detached from tissue culture plates using 1 mg EDTA in PBS, washed once in PBS, and resuspended in serum-free medium at a concentration of 10$^5$ cells/ml except for the Mn$^{2+}$ activation assays. 100 µl of the suspension was added to each coated well. After a 45-min incubation at 37 °C, the plates were placed on an orbital shaker and pulsed three times for 10 s at 300 rpm to remove the nonadherent cells. The wells were then washed with PBS once, and 60 µl of 7.5 mM p-nitrophenyl-N-acetyl-β-D-galactosaminide (Sigma) was added in a buffer containing 0.1% Triton X-100. After a 6-h incubation, 90 µl of developer solution was added (50 mM glycine, 5 mM EDTA, pH 10.4), and the absorbance was read at 410 nm. In parallel, 100 µl of the cell suspension was centrifuged, and the pellet was resuspended in 60 µl of p-nitrophenyl-N-acetyl-β-D-galactosaminide solution as the 100% cell control. The percentage of adhesion was expressed as the ratio between the mean absorbance of three wells and the absorbance of the 100% cell control. To analyze the effect of Mn$^{2+}$ on cell adhesion, cells were detached from dishes, washed with 1 mM EDTA and calcium-, magnesium-free Tyrode’s buffer (150 mM NaCl, 2.5 mM KCl, 12 mM NaCO$_3$, 2% BSA, pH 7.4), and then resuspended in the calcium-, magnesium-free Tyrode buffer at 6 × 10$^5$ cells/ml. 50 µl of cell suspension were mixed with an equal volume of MnCl$_2$ at the desired concentration. Cells were then plated, and the standard adhesion assay was performed as described above.

Migration Assay—Migration was assayed using a transwell assay as described previously (28). Transwells were coated with fibronectin at different concentrations for 2 h followed by a 1-h incubation with 2% BSA. Cells were detached with 1 mg EDTA in PBS, washed once with PBS, and resuspended in serum-free hybridoma medium CCMI (Hy- cern Laboratories, Inc., Logan, UT). 1 × 10$^5$ cells were added to each well. After a 3-h incubation at 37 °C, the cells remaining on the top membrane were carefully removed using a cotton swab. The cells that had migrated cells onto the lower surface of the membrane were fixed with methanol and stained with methylene blue for 30 min. Cells on the lower surface of the membrane were counted under a phase-contrast microscope. Six fields were scored for each well. The numbers of cells that had migrated in each field were expressed as the mean of two sets of wells.

Cell Spreading—Cell spreading assays were performed as described previously (29, 37). 96-well, non-tissue culture plates were coated with 10 µg/ml fibronectin. Cells were detached, washed as described above for adhesion assays, and then resuspended in serum-free medium at 2 × 10$^5$ cells/ml. 50-µl aliquots of cell suspension were mixed with an equal volume of antibody solution and added to the fibronectin-coated wells. The cells were incubated for 1 h at 37 °C and fixed in 3% formaldehyde, and then six random fields were scored using a phase-contrast microscope for spreading as described previously (29). Only cells with fully formed lamina were counted as spread cells. The percentage of spreading was determined from the ratio of the number of spread cells to the total number of cells that had migrated in each field were expressed as the mean of two sets of wells.

Fluorescence Staining—12-mm glass coverslips (Fisher) were coated with different fibronectin concentrations. Cells were detached from plates using 0.2 g/ml EDTA in PBS and resuspended in serum-free medium (minimal essential medium with 2% BSA). 2.5 × 10$^5$ cells were added to each coverslip and incubated at 37 °C for 3 h. The coverslips were washed twice with PBS, fixed with 3% formaldehyde in PBS, and
permeabilized with 0.2% Triton X-100 for 10 min. The cells were incubated with primary antibody (20 μg/ml in blocking buffer) for 30 min followed by incubation with FITC-conjugated sheep anti-mouse IgG. Coverslips were blocked with blocking buffer (5% horse serum in PBS) before each incubation and then washed extensively. The coverslips were mounted, and the cells were viewed on an Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

**Cell Extracts and Western Blotting**—Cells were prepared as described above for adhesion assays. 1.5 × 10^6 cells were added to 60-mm fibronectin-coated dishes. After a 3-h incubation at 37°C, cells were lysed in 100 μl of cold radioimmuno precipitation extraction buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40). The pellet was re-suspended in 20 mM EDTA. The lysate was clarified by centrifugation for 15 min followed by centrifugation at 14,000 × g for 5 min. Protein concentration was determined using the bichinchoninic acid (Pierce) method. The appropriate volume of 4× protein loading buffer (0.25 mM Tris-HCl, 8% SDS, 40% glycerol, 0.1% bromphenol blue, 20% 2-mercaptoethanol) was added to 30 μg of protein. The sample was then immersed in boiling water for 5–10 min, loaded on a 12% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was blocked with 1% BSA in TST buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40, 0.1% Tween 20) for 1 h. The PY20 antibody was diluted to 1:1000 in blocking buffer and incubated with the membrane for 1 h. After a TST buffer washing, the PY20 blot was incubated for 30 min with a horseradish peroxidase-conjugated antibody (diluted to 1:10,000 in blocking buffer) followed by a washing with TST buffer. The blot was visualized by chemiluminescence (Pierce Super Signal™, Pierce) using x-ray film (Eastman Kodak Co.).

**Fibronectin Binding Assay**—Fibronectin was first conjugated with FITC as follows. The fibronectin was diluted to a concentration of 0.5 mg/ml in carbonate saline buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, 0.15 M NaCl), and FITC was dissolved in the same buffer at 0.5 mg/ml. 50 μl of the FITC solution was added to 0.5 mg of fibronectin, and the mixture was incubated at room temperature for 1 h. The free FITC was removed using a Sephadex G-25 column equilibrated with PBS. The concentration of the collected FITC-labeled fibronectin was calculated by the equation [FITC-fibronectin] = (A₄₅₀ – 0.35 × A₄₉₅)/V x 1.23. A₄₅₀ and A₄₉₅ are the absorbance of FITC-fibronectin at 280 and 495 nm, respectively. 1.23 is the extinction coefficient of the FITC-labeled fibronectin. (30). The fibronectin binding assay was performed as follows. Cells were detached from plates using 0.2 g/ml EDTA in CMP-HH buffer and washed twice with serum-free medium. Cells were then blocked with 2% BSA in minimal essential medium for 30 min and incubated with different concentrations of FITC-fibronectin for 30 min at room temperature. Cells were analyzed by flow cytometry as described above. The amount of fibronectin bound was estimated from the fluorescence intensity of the cells. The specific binding was obtained by subtracting the background binding (the fluorescence intensity of the untransfected CHO B2 cells) from the total binding of the transfectants. The data were analyzed using a Scatchard plot (31). The ratio of the bound to the free concentration of fibronectin was plotted as a function of the bound concentration of fibronectin. The data were fit to a straight line, and the slope was used to estimate the apparent dissociation constant (Kᵰ). These were the apparent dissociation constants determined using the wild type and mutant α₅ subunits as the ligand.

**Materials**—The VIF4, VD1, HA1, and VD10 mAbs directed against human α₅ integrins were gifts from Dr. Ralph Isberg (Tufts University, Boston, MA), and BIID2 mAb was a gift from Dr. C. Damasky (University of California, San Francisco, CA). The mAbs 16G3 and 3B8 were from Dr. K. Yamada (NCI, National Institutes of Health, Bethesda, MD). The anti-phosphotyrosine mAb, PY20, was purchased from Transduction Laboratories (Lexington, KY). The anti-vinulin mAb, FITC, and poly-l-lysine were purchased from Sigma. Transwells were purchased from Corning Costar Co. (Cambridge, MA). Fibronectin for the soluble ligand assay was a gift from Dr. Deane Mosher (University of Wisconsin, Madison, WI). The fibronectin used for other assays was purified from human plasma by affinity chromatography as described elsewhere (61). The human α₅ and chicken α₅ cDNA were kindly provided by Dr. L. Reichardt (University of California, San Francisco, CA). The CHO B2 cell line was a gift from Dr. R. L. Juliano (University of North Carolina, Chapel Hill, NC).

**RESULTS**

**Construction and Expression of α₅ Chimeras**—Previous publications suggest that the N-terminal portion of some α sub-units is involved in integrin-ligand interaction. For our studies, we selected a 144-residue fragment on the α₅ subunit as the putative ligand-binding region (Fig. 1). This region encompasses two cation-binding motifs. It is homologous to the putative ligand-binding site, which cross-links to a radiolabeled GRGDS PK peptide on the α₅ subunit (16). It also shares sequence homology with the putative ligand interaction sites on the α₁β₁ and α₁β₁ subunits, respectively (20, 21). Chou-Fasman (32) and Rosbon-Garnier folding algorithms (33, 34) predict loop structures in this region (data not shown). The region was divided into four smaller fragments. These were based on the known exon-intron boundaries on integrin α subunits and short nonhomologous peptide regions surrounding by sequences of high homology. To preserve the structure, the four segments, designated as domains I, II, III, and IV on the α₅ integrin, were exchanged with their counterparts on chicken α₅ subunit (Table I). The α₅ subunit was chosen because it also dimerizes with the β₁ subunit but has a different ligand specificity.

The wild type and chimeric α₅ subunits, designated as α₅I, α₅II, α₅III, and α₅IV, were transfected into the CHO B2 cell line, which expresses minimal endogenous α₅ integrin (25). Cells were grown in selective medium to achieve stable transfectants and then sorted by flow cytometry for those expressing similar levels of α₅. Neither the α₅IL nor α₅IV could be detected on the cell surface following either transient or stable transfections (Table I). This was seen in multiple, independent transfections. In contrast, both α₅III and α₅IV express on the cell surface. Cells expressing comparable levels of wild type and mutant α₅ were collected by flow cytometry (Fig. 2). Swapping the four subdomains in different combinations, including domains III and IV, did not result in proteins expressed on the cell surface.

**CHO B2 Cells Expressing α₅III and α₅IV Chimeras Exhibit Impaired Adhesion, Spreading, and Migration**—CHO B2 cells expressing chimeric integrins showed impaired adhesion when compared with wild type transfectants (Fig. 9). Whereas wild type α₅ transfected CHO B2 cells readily attach to fibronectin, the adhesion of α₅III and α₅IV-transfected cells was significantly reduced (p < 0.05). Untransfected CHO B2 cells showed minimal adhesion. The adhesion observed in the mutants was not reversed by plating cells on increased fibronectin concentrations. The adhesion of the α₅ transfectants to fibronectin was specifically mediated by exogenous α₅, since adhesion-perturbing antibodies specific for human α₅ integrin inhibited adhesion (data not shown).

CHO B2 cells transfected with chimeric α₅ subunits also

![FIG. 1. A diagram of the chimeric α₅ subunits used in this study. A schematic model of the α₅ integrin is shown. A 144-residue region near the first and second cation binding motifs, which are represented by the thick bars, was selected and divided into four segments (indicated as I, II, III, and IV). Residues at the boundaries between segments are shown. Each segment was exchanged with its counterpart on the α₅ subunit.](image-url)
TABLE I

| Domain(s) swapped | Residues replaced on α5 | Counterpart from α6 | Expression on CHO B2 |
|-------------------|-------------------------|---------------------|----------------------|
| I                 | Gly223–Ser275           | Ala301–Ser275       | No expression |
| II                | Val276–Ala323           | Leu326–Ala323       | No expression |
| III               | Ser224–Ala276           | Ser224–Ala276       | Expressed |
| IV                | Pro248–Val367           | Pro248–Ile367       | Expressed |
| I and II          | Gly223–Met322           | Ala301–Met322       | No expression |
| II and III        | Val276–Ala347           | Leu326–Ala347       | No expression |
| III and IV        | Ala223–Val367           | Ser224–Ile347       | No expression |
| I, II, and III    | Gly223–Ala347           | Ala301–Ala331       | No expression |

Fig. 3. Adhesion of α(III) and α(IV) chimeras to fibronectin. 96-well plates were coated with fibronectin at different concentrations. 3 x 10⁴ cells were plated in each well and incubated for 45 min, and the weakly adherent and nonadherent cells were removed by shaking the plates on an orbital shaker (see “Experimental Procedures”). The adherent cells were counted by a colorimetric assay. The results are expressed as the percentage of cells that adhered relative to the total number of cells added to the well. 100% is where all of the added cells adhere. Error bars represent the S.D. G, wild type α, α, untransfected CHO B2 cells; D, α(III) x α(IV).

Fig. 4. Morphology of cells expressing wild type and chimeric α5β1 on cell surface. CHO B2 cells expressing wild type or mutant α5 were stained with the primary mAb, VIF4, which recognizes the human α5 integrin subunit, followed by FITC-sheep anti-mouse IgG. Cells were selected for similar expression levels by fluorescence-activated cell sorting.

showed altered spreading. After 1 h of incubation on fibronectin, most of the wild type α5 transfectants were fully spread. In contrast, only a small fraction of the α(III) and α(IV)-transfected cells were spread (Fig. 4). Most remained round even on high concentrations of fibronectin (data not shown). The differences in spreading were still apparent after longer incubation times both in the presence and absence of serum.

Migration of CHO B2 cells transfected with the α6 chimera was assayed using a transwell assay. The migration of α(III) and α(IV) was substantially lower than that of the wild type α5 transfectant on either high or low concentrations of fibronectin (Fig. 5). α(IV)-expressing cells migrated less than α(III)-expressing cells.

Cells Expressing α5 Chimeras Show Small Alterations in Their Affinity for Fibronectin—Two straightforward mechanisms can explain the differences in adhesion, spreading, and migration observed in the wild type and chimeric α5 receptors. 1) Domains III and IV may be directly involved in ligand binding by either interacting with fibronectin or providing critical conformational support for the ligand-binding pocket. Mutations in this region, then, would lead to a significant decrease in receptor affinity for fibronectin. 2) Domains III and IV mediate a ligand-induced transmembrane signal transduction event that affects cytoplasmic function. Perturbing this pathway would lead to inhibited signal propagation, which would affect integrin avidity and spreading.

The first hypothesis was investigated by assaying the binding of soluble fibronectin to cells expressing wild type and chimeric α5 subunits. Cells were incubated with different concentrations of purified FITC-fibronectin, and binding was assayed by flow cytometry. The binding could be inhibited by an adhesion-perturbing antibody, which demonstrates the specificity of the interaction. The data were analyzed using a Scat-
The RGD binding domain on wild type fibronectin. The affinity of the RGD sequence. Spreading and adhesion of the wild type and mutant is large and persistent even at higher fibronectin concentrations. The affinity hypothesis was investigated further using adhesion-inhibiting antibodies and peptides. The $\alpha_5\beta_1$ integrin interacts with fibronectin at two sites: the main (RGD, Arg-Gly-Asp) and the synergy (PHSRN, Phe-His-Ser-Arg-Asn) sites (36). 16G3 is an anti-fibronectin mAb that recognizes the RGD sequence and inhibits its interaction with the $\alpha_5\beta_1$ integrin (37). If the RGD binding domain on $\alpha_5\beta_1$ was altered by our swap mutations, the interaction between fibronectin and $\alpha_{5I\!I\!I}\beta_1$ or $\alpha_{5I\!V}\beta_1$ would be insensitive to 16G3 and independent of the RGD sequence. Spreading and adhesion of the wild type and chimeric $\alpha_5$ transfectants to fibronectin was inhibited by both the 16G3 mAb (Fig. 7) and the presence of the GRGDSP peptide (Fig. 8). The chimeras were also more rounded in the presence of the peptide. This inhibition was specific, since the control peptide, GREGESP, did not similarly affect cell adhesion (not shown). It is significant that the dose dependence of the GRGDSP inhibition of attachment was similar for both the wild type $\alpha_5$ and the $\alpha_{5I\!I\!I}$ and $\alpha_{5I\!V}$ chimeras. This shows that the RGD-binding site is intact and indicates that its affinity is not grossly altered.

Another anti-fibronectin mAb, 3B8, inhibits the interaction with $\alpha_5\beta_1$ and the PHSRN synergy site (37). Using the rationale described above, we used this mAb to determine whether 3B8 inhibited recognition of the PHSRN site on fibronectin (Fig. 7). As reported above for the RGD site, the 3B8 mAb inhibited the spreading of CHO cells expressing either the wild type or chimeric $\alpha_5$ receptors. The antibody concentration required for inhibition was similar in the cells expressing the $\alpha_5$ and chimeric receptors. These data suggest that the PHSRN-binding site, like that for RGD, on the chimeric $\alpha_5$ subunit is not grossly affected.

We also investigated whether the chimeras produced gross conformational changes in the chimeric $\alpha_5$ subunits. A panel of four adhesion-perturbing mAbs all inhibited, with similar concentration optima, the adhesion of cells expressing wild type or chimeric $\alpha_5$ receptors. A non-adhesion-perturbing mAb, 6F4, was assayed for binding at different concentrations; both the mutants and wild type $\alpha_5$ showed similar concentration optima (data not shown). As discussed above, most of the mutations we constructed resulted in subunits that did not appear on the cell surface, suggesting a constrained conformation in this region. Thus, this observation along with the antibody binding and concentration dependence for inhibition of adhesion supports the view that the chimeras do not exhibit gross conformational alterations.

Finally, we investigated the divalent cation requirement for...
adhesion to fibronectin. Integrins have several sites that bind divalent ions, which are critical for ligand binding. We analyzed the effects of Mn$^{2+}$, an activator of integrin binding, on adhesion to fibronectin. The effect of Mn$^{2+}$ on cells expressing either the wild type or $\alpha_5$ chimera is similar (Fig. 9); i.e. the adhesion of cells expressing either the wild type or mutant $\alpha_5$ is enhanced by Mn$^{2+}$ and shows a similar concentration dependence. Thus, the mutations do not cause a gross change in the interactions with divalent cations.

adhesion to fibronectin. The effect of Mn$^{2+}$ spreading of a enhanced by Mn$^{2+}$ the synergy site for 10 min. 1 turbs adhesion via the RGD site, or the 3B8 mAb, which interacts with

96-well plates were coated with 20 $\mu$g/ml fibronectin. Cells were resuspended at a density of $6 \times 10^5$ cells/ml. 50 $\mu$L of the cell suspension was mixed with an equal volume of MnCl$_2$ at the designated concentration and plated into each well. After a 45-min incubation, unbound and weakly adherent cells were removed by shaking, and the remaining adherent cells were quantitated by a colorimetric reaction as described under “Experimental Procedures.” The result is expressed as the percentage of the initially plated cells that adhere. ○, wild type $\alpha_5$; □, $\alpha_5$III; ▯, $\alpha_5$IV.

Taken together, our data suggest that domains III and IV are most likely not directly involved in ligand binding. Although the mutations in these domains caused small alterations of the binding affinity in the chimeric receptors, the perturbations do not readily explain the large differences observed in migration and spreading. This notion is further supported by the similar fibronectin concentration dependence of adhesion and spreading for both wild type and chimeric $\alpha_5$ subunits.

Cells Expressing the $\alpha_5$ Chimeras Show Inhibited Focal Adhesion Formation and Tyrosine Phosphorylation—In the absence of clear evidence for major alterations of ligand binding, an alternative hypothesis was investigated, i.e. that the chimeras exhibited inhibited ligand-induced signal transduction and/or focal adhesion formation. The latter was assayed by plating cells on fibronectin for 4 h and staining with an antivinculin antibody (Fig. 10). CHO B2 cells expressing the wild type $\alpha_5$ spread on the substrate and formed distinct focal adhesions at the cell periphery. Untransfected cells remained rounded, and no prominent focal adhesions were observed. A large proportion of $\alpha_5$III- and $\alpha_5$IV-transfected cells, although adherent, were not well spread and formed only a few, weakly staining focal adhesions.

Since focal adhesions are hot spots for tyrosine phosphorylation, a major event that is initiated by cell attachment, we stained for phosphotyrosine in focal adhesions using PY20, a mAb specific for phosphotyrosine residues (Fig. 11). As reported above with vinculin staining, cells expressing the chimeras showed weak phosphotyrosine staining that was not organized into focal adhesion-like structures. In contrast, cells expressing wild type $\alpha_5$ stained brightly and localized in focal adhesions. This could result from inhibited focal adhesion formation or from inhibited tyrosine phosphorylation. We assayed for phosphorylation of cellular proteins by incubating cells on fibronectin for 3 h and immunoblotting extracts using PY20 (Fig. 12). Large differences in the tyrosine phosphorylation pattern were observed between wild type $\alpha_5$ and $\alpha_5$III and $\alpha_5$IV mutants. In wild type $\alpha_5$ transfectants, prominent tyrosine-phosphorylated proteins were evident at $\sim$125 and $\sim$70 kDa after incubation on fibronectin. There was little increase in

FIG. 7. Effect of RGD and synergy site-perturbing mAbs on the spreading of $\alpha_5$ transfectants. 96-well plates were coated with 20 $\mu$g/ml fibronectin. Wells were incubated with 16G3 mAb, which perturbs adhesion via the RGD site, or the 3B8 mAb, which interacts with the synergy site for 10 min. 1 $\times$ 10$^4$ cells were then plated into the each well. After a 1-h incubation, cells were fixed and scored using a phase-contrast microscope as described under “Experimental Procedures.” The number of spread cells (see “Experimental Procedures”) and number of total cells in each view field were counted. The percentage of spreading was from the ratio of the two numbers. □, wild type $\alpha_5$; □, $\alpha_5$III; ▯, $\alpha_5$IV.

FIG. 8. Effect of the GRGDSP peptide on the adhesion of $\alpha_5$ transfectants to fibronectin. 96-well plates were coated with 20 $\mu$g/ml fibronectin. Cells were resuspended at a density of $6 \times 10^5$ cells/ml. 50 $\mu$L of cell suspension was mixed with an equal volume of GRGDSP peptide and plated into each well. After a 45-min incubation, unbound and weakly adherent cells were removed by shaking, and the remaining adherent cells were quantitated by a colorimetric reaction as described under “Experimental Procedures.” The result is expressed as the percentage of the initially plated cells that adhere. ○, wild type $\alpha_5$; □, $\alpha_5$III; ▯, $\alpha_5$IV.

FIG. 9. Effect of Mn$^{2+}$ on adhesion to fibronectin. 96-well plates were coated with 20 $\mu$g/ml fibronectin. Cells were resuspended at a density of $6 \times 10^5$ cells/ml. 50 $\mu$L of the cell suspension was mixed with an equal volume of MnCl$_2$ at the designated concentration and plated into each well. After a 1-h incubation, cells were fixed and scored using a phase-contrast microscope as described under “Experimental Procedures.” The result is expressed as the percentage of the initially plated cells that are bound. 100% is when all of the cells adhere. ○, wild type $\alpha_5$; □, $\alpha_5$III; ▯, $\alpha_5$IV.

Since focal adhesions are hot spots for tyrosine phosphorylation, a major event that is initiated by cell attachment, we stained for phosphotyrosine in focal adhesions using PY20, a mAb specific for phosphotyrosine residues (Fig. 11). As reported above with vinculin staining, cells expressing the chimeras showed weak phosphotyrosine staining that was not organized into focal adhesion-like structures. In contrast, cells expressing wild type $\alpha_5$ stained brightly and localized in focal adhesions. This could result from inhibited focal adhesion formation or from inhibited tyrosine phosphorylation. We assayed for phosphorylation of cellular proteins by incubating cells on fibronectin for 3 h and immunoblotting extracts using PY20 (Fig. 12). Large differences in the tyrosine phosphorylation pattern were observed between wild type $\alpha_5$ and $\alpha_5$III and $\alpha_5$IV mutants. In wild type $\alpha_5$ transfectants, prominent tyrosine-phosphorylated proteins were evident at $\sim$125 and $\sim$70 kDa after incubation on fibronectin. There was little increase in...
tyrosine phosphorylation in CHO B2 cells plated onto fibronectin. Protein phosphorylation in $\alpha_5\beta_1$ and $\alpha_5\beta_4$-transfected cells was also weak upon adhesion to fibronectin. The decreased protein phosphorylation seen in $\alpha_5\beta_4$ was larger than that seen with $\alpha_5\beta_1$.

In summary, CHO B2 cells expressing $\alpha_5\beta_1$ and $\alpha_5\beta_4$ chimeras showed significantly reduced phosphorylation on tyrosine and inhibited focal adhesion formation. This points to inhibited transmembrane signal propagation as a likely effect of the chimeric $\alpha_5$ subunits.

Role of the $\alpha_5$ Subunit Cytoplasmic Domains in Focal Adhesion Formation—Post-receptor occupancy events, including association with focal adhesion components and tyrosine phosphorylations, are regulated by the outside-in signal propagation, which is thought to involve a conformational change in the cytoplasmic domain. Considerable evidence supports the view that the $\beta$ subunit cytoplasmic domain is the major functional moiety. It has binding sites for several focal adhesion molecules including focal adhesion kinase, a tyrosine kinase, and isolated $\beta_1$ cytoplasmic domains (fused to receptors like interleukin-2) localize to focal adhesions (8, 38–42). In this view, the $\alpha$ subunit cytoplasmic domain regulates integrin function by inhibiting interactions of focal adhesion components with the $\beta$ subunit cytoplasmic domain. We hypothesized that removal of the $\alpha_5$ cytoplasmic domain from our chimeric mutants would free the $\beta_1$ subunit and restore spreading and tyrosine phosphorylation. Therefore, we deleted the cytoplasmic domains on the $\alpha_5\beta_1$ and $\alpha_5\beta_4$ chimeras, which is present on most $\alpha$ subunits. The new chimeras, designated as $\alpha_5\beta_1cyto$ and $\alpha_5\beta_4cyto$, were transfected into CHO B2 cells. The deletion resulted in unstable expression of $\alpha_5\beta_1cyto$, $\alpha_5\beta_4cyto$ expressed stably and was selected for similar expression levels. The cells were assayed for adhesion, spreading, migration, and focal adhesion formation. As summarized in Table II, the phenotypes observed with the new chimera were indistinguishable from that of the chimeras with an intact $\alpha$ subunit cytoplasmic domain. Thus, cells expressing chimeric integrins are unable to form focal adhesions either in the presence or absence of a complete $\alpha$ subunit cytoplasmic domain. This contrasts with the behavior of receptors with interleukin-2 chimeras containing isolated $\beta_1$ subunit cytoplasmic domains, which associate with preformed focal adhesions.

**DISCUSSION**

In this study, we identified domains on the integrin $\alpha_5$ subunit that mediate cell spreading and signaling on fibronectin. Genetic approaches to identifying functional regions on integrin $\alpha$ subunits have been frustrated by the sensitivity of their extracellular domains to mutation. Such mutant $\alpha$ subunits often do not dimerize or appear on the surface (19). To avoid this, we divided a putative ligand binding region on the integrin $\alpha_5$ subunit into four smaller segments that are flanked by conserved sequences and form predicted loops. We made chimeric $\alpha_5$ subunits by substituting each segment with its counterpart on the $\alpha_6$ subunit. Since the $\alpha_5$ and $\alpha_6$ subunits dimerize with the same $\beta_1$ subunit, the $\alpha_5$ chimeras should minimize conformational perturbations. Two of the $\alpha_5$ chimeras that we constructed express on the cell surface. Both contain swaps near putative cation binding regions. When introduced into the CHO B2 cell line, which is deficient in expression of endogenous $\alpha_5$ integrin, the cells showed inhibited spreading, adhe-
sion, migration, and focal adhesion formation when compared with CHO B2 cells expressing the wild type α5 subunit. However, the affinity of the mutants for binding to fibronectin decreased only 3-fold. The recognition sites for RGD and PHSRN were not grossly perturbed on the chimeric α5 subunits, since RGD peptides and antibodies directed against both the RGD and PHSRN sites inhibited adhesion. Metal ions still promoted cell adhesion at concentrations similar to those for the wild type α5 subunit. These data suggest that the defects most likely do not arise solely from alterations in receptor affinity. However, as indicated by phosphotyrosine blotting and inhibited spreading, signal propagation mediated by α5 was disrupted by the mutations, suggesting a model in which the chimeras inhibit the putative conformation changes that mediate ligand-induced transmembrane signal propagation.

It has been previously reported that the N-terminal one-third of integrin α and β subunits mediate integrin-ligand recognition (9). This region is also involved in the interactions between α and β subunits. Despite the conservative nature of our mutations, it is interesting that most of the chimeric α5 subunits that we made failed to express on cell surface. Furthermore, even some conserved point mutations in the EF-hands, such as D284E, D334E, N336E, D338E, and G398S, also resulted in no detectable surface expression (data not shown). Similar inhibitions of dimerization and surface expression in mutant α subunits have been reported by others (19). These observations all point to a high conformational sensitivity in this region. Presumably, the encompassed cation-binding motifs are crucial for proper folding and/or dimerization.

The altered spreading, migration, and focal adhesion formation observed with CHO B2 cells expressing the α5III and α5IV do not likely arise from major conformational changes. As pointed out above, any major conformational alteration in this tightly folded structure would probably abolish its surface expression. In our experiments, both α5III and α5IV express on the cell surface. Furthermore, cells transfected with either the wild type or mutant α5 subunits showed similar concentration optima for antibody binding and inhibition of adhesion, indicating that the mutations do not grossly alter the affinity of α5III and α5IV to several antibodies.

Our data also suggest that the interaction of the chimeric α5 receptors with fibronectin is perturbed only about 3-fold. Both the RGD and PHSRN sequences, which are the important motifs on fibronectin for adhesion to fibronectin, are also not grossly altered. Adhesion-perturbing antibodies directed against these sites on fibronectin inhibit the adhesion and spreading of both wild type and mutant cells. Furthermore, RGD-containing peptides inhibit adhesion of chimeric integrins at concentrations that are similar to those that inhibit adhesion of the wild type subunit. In addition, increasing the substrate concentration did not enhance adhesion, spreading, or migration in cells expressing the chimeric α5 subunits. Mass action predicts that decreased affinity would be compensated for by an increase in substrate concentration as observed previously for migration and spreading (28, 43, 44). Taken together, these results demonstrate that major recognition sites on α5III and α5IV are intact.

It is unlikely that the inhibited adhesion and spreading arise solely from the 3-fold affinity alteration caused by the mutations. The evidence for this conclusion includes the following. First, the affinity change is much smaller than that observed for spreading, migration, and focal adhesion formation. A swap mutation on the β3 subunit has been reported that increases the affinity of integrin αIIbβ3 to fibrinogen over 30-fold. However, the adhesion of the mutant and the wild type cells to fibronectin, while different on low concentrations of substrate, showed no difference at higher concentrations (35). Cells transfected with αIIb, when assayed in the presence of LIBS6, an activating antibody, showed a 10-fold increased affinity for fibrinogen; however, the spreading of the cells was similar to those not treated with LIBS6. Second, it has been shown previously that some mutations affect cell adhesion and spreading without altering the ligand binding affinity. Mutations within the divalent cation-binding regions of integrin α4, for example, alter cell adhesion significantly but have no effect on the binding of soluble VCAM-1 (45). Another mutant in the region outside of the cation-binding region also showed inhibited adhesion while still bound to VCAM-1 (46). Thus, changes in cell adhesion and spreading do necessarily arise from large affinity changes. Conversely, small affinity alterations also do not necessarily lead to impaired cell spreading. Third, recent publications (28, 43, 44) show that alterations in receptor affinity shift the concentration optimum for cell migration in a manner predictable by force and mass action considerations. In these studies, 3-fold changes in receptor affinity would not show the magnitude and type of inhibitions of migration and spreading seen in cells expressing the chimeric integrins. In this context, it is particularly important to note that the inhibition of migration, for example, cannot be compensated for by increased substrate fibronectin concentration. Thus, although the affinity change of α5III and α5IV chimeras to fibronectin may contribute to the altered spreading, migration, and focal adhesion formation of the chimera, it is unlikely that they are the primary contributors. However, since adhesion is a highly cooperative process, we cannot rigorously exclude a contribution from the relatively small change in affinity.

Alterations in a post-ligand-binding event provide a more likely explanation for the defects in the chimeric α5 subunits, since we observed inhibition of well characterized post-ligand-binding processes. Adhesion mediated by the α5 integrin induces spreading, focal adhesion formation, and phosphorylation of several prominent proteins migrating in the molecular mass area of 130 and 70 kDa. All of these were inhibited in CHO B2 cells expressing the chimeric integrins. Therefore, we hypothesize that the chimeras disrupt ligand-initiated signal propagation.

The mechanism by which ligand binding to integrins initiates a signal transduction process that propagates from the extracellular to the cytoplasmic side of the membrane is not known. Considerable evidence points to a major, long range conformation change involving cation binding regions in the integrin molecule as a key event in this process (47, 48). Ligand binding to the αIIbβ3 integrin, for example, induces a conformation that is recognized by certain mAbs (49). PAC-1 is a mAb directed against the β3 integrin subunit that recognizes an active conformation of αIIbβ3. The affinity of the PAC-1 mAb for αIIbβ3 increases 20-fold following the addition of peptide li-

| Effect of cytoplasmic deletions on α5, α5III, and α5IV phenotypes | α5 | α5III | α5IV | α5III/IV |
|---|---|---|---|---|
| Adhesion | + | - | - | - |
| Spreading | + | - | - | - |
| Migration | + | + | + | + |
| Focal adhesion formation | + | + | + | + |

2 A. Huttenlocher, unpublished data.
gands (50). This change has been hypothesized to propagate to the cytoplasmic domain via a “hinge” where the membrane proximal regions of the α and β subunit cytoplasmic domains reside.

The regions swapped in our chimeras represent regions in or near putative cation binding motifs. Our domain III encompasses a cation-binding site, while domain IV is adjacent to one. These regions are sensitive to conformational changes and implicated in regulating the affinity of integrin-ligand binding (22, 51, 52). Changes in the cations bound to integrins alter the affinity of several integrins and can expose new epitopes. Replacing Ca$^{2+}$ with Mn$^{2+}$, for example, increases the affinity of several different integrins, while the binding of Mg$^{2+}$ exposes an epitope on integrin α5β3 (22, 51, 52). The affinity of integrins can also be affected through alterations of the cytoplasmic domains or by signals from the cytoplasm (53, 54). Thus, conformational information appears to travel reciprocally from the cytoplasmic domains to the ligand-binding site and vice versa. The cation binding domain appears to lie on this pathway.

How might the domains III and IV participate in the conformation changes that mediate ligand-initiated signal transduction? The β-propeller model provides potential insights (15). In this model, the N-terminal region of the integrin α subunit folds into a β-propeller domain, which is composed of seven four-stranded β-sheet structures. It has been proposed that integrins bind to ligands and a putative Mg$^{2+}$ ion at the upper face of the propeller. The Ca$^{2+}$ binding motifs are predicted to be on the lower face of the propeller. Domain III in our study encompasses strand 1 and part of strand 2 of the fifth β-sheet structure and is predicted to interact with Ca$^{2+}$ at the lower face of the propeller. Domain IV contains part of strands 2 and 3 of the fifth β-sheet and is located on top of the propeller, possibly at or close to the interface between the ligand and the integrin. One hypothesis is that when α5β3 interacts with fibronectin, domain IV, which is adjacent to the ligand binding pocket, undergoes a conformational change and causes a change in domain III, which in turn propagates the alteration toward the cytoplasmic domain. The metal ion associated with domain III helps to stabilize the proper conformation or assist in the transformation. Domains III and IV therefore constitute a mediator that directs the signal from ligand-recognition site to the cytoplasm. Mutations in α3III and α5IV would disrupt such a pathway and result in attenuated signaling. It is likely that the signal-propagation domain(s) would be coupled to the ligand-binding domain in the sterically constrained environment that appear to characterize integrin extracellular domains. Considering the proximity of domains III and IV to the ligand-binding site, mutations in this region may also cause a small conformational alteration of the ligand-binding pocket, which could produce the 3-fold effects that we observe.

A related hypothesis is that domains III and IV reside at or adjacent to the interface between the α and β subunits. It is known that ligands and divalent cations induce a conformational change in the integrin heterodimer, and the interaction between the α and β subunit may be involved in this conformational change. In the model discussed above, the β-propeller domain on the α subunit interacts with the β subunit (55). It has also been reported that α5β3 subunits dissociate when the Ca$^{2+}$ is removed (56). In this context, domains III and IV in our study may be part of the interface between the subunits. In this location, the α5IIIβ1 and α5IVβ1 chimeras would inhibit intersubunit conformation changes that mediate ligand-initiated signal transduction.

The mechanisms by which ligand binding to the extracellular domain couples to the cytoplasmic responses that mediate adhesion, spreading, and tyrosine phosphorylation are an enigma. The available evidence points to ligand occupancy and receptor aggregation being required for a complete response (38, 39, 57). Ligand binding alone is sufficient to target integrins to focal adhesions but probably not to assemble them (39). Similarly, receptor aggregation alone, while sufficient to promote the association of a large number of signaling molecules, does not lead to focal adhesion formation. Focal adhesion assembly appears to require both ligation and aggregation (58). Considerable evidence also points to the cytoplasmic domain of several integrin β subunits as critical for both integrin-mediated tyrosine phosphorylation and association with focal adhesion molecules. The α subunits, in turn, appear to modulate the functionality of the β subunit cytoplasmic domain, perhaps through a steric interaction (59, 60). In this context, it is interesting that removing the α5 cytoplasmic domain from the mutants does not alter the phenotype of the α5IV chimera. It underscores a difference between the ability to localize in focal adhesions and to form or organize focal adhesions. We can only speculate on why our mutations alter the ability to organize focal adhesions. Perhaps ligand binding in the mutants results in inhibited receptor aggregation or accessibility of the β5 cytoplasmic domain. In either case, these mutants should be very useful for understanding the roles of α5 subunit structure and function in adhesion-induced signaling in a variety of cellular processes.

Acknowledgments—We thank R. Juliano for providing CHO B2 cell line, D. Mosher for fibronectin, and L. Reichardt for the α5 and α6 cDNAs. We also thank M. Humphries for useful discussions, M. Ware for comments on the manuscript, Z. Zhang for discussion on fibronectin binding, and Margot Lakonishok for advice throughout the study.

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