Integrins mediate signal transduction through interaction with multiple cellular or extracellular matrix ligands. Integrin αβ3 recognizes fibrinogen, von Willebrand factor, and vitronectin, while αβ1 does not. We studied the mechanisms for defining ligand specificity of these integrins by swapping the highly diverse sequences in the I domain-like structure of the β1 and β3 subunits. When the sequence CTSEQNC (residues 187–193) of β1 is replaced with the corresponding CYD-MKTC sequence of β3, the ligand specificity of αβ1 is altered. The mutant (αβ1-3-1), like αβ3, recognizes fibrinogen, von Willebrand factor, and vitronectin (a gain-of-function effect). The αβ1-3-1 mutant is recruited to focal contacts on fibrinogen and vitronectin, suggesting that the mutant transduces intracellular signals on adhesion. The reciprocal β3-1-3 mutation blocks binding of αβ3 to these multiple ligands and to LM609, a function-blocking anti-αβ3 antibody. These results suggest that the highly divergent sequence is a key determinant of integrin ligand specificity. Also, the data support a recent hypothetical model of the I domain of β1, in which the sequence is located in the ligand binding site.

Integrins are a family of α/β heterodimers of cell adhesion receptors that mediate cell-extracellular matrix and cell-cell interactions (1–5). Integrin-ligand interactions are critically involved in the pathogenesis of many diseases in human and animal models. Although integrin-ligand interaction is a therapeutic target, we poorly understand at the molecular level how integrins recognize multiple ligands. Evidence suggests that the I or A domain, a set of inserted sequences consisting of about 200 amino acid residues, of several integrin subunits (αM, αL, α1, α2) is important in ligand binding and receptor activation (reviewed in Ref. 6 and references therein). The presence of an I domain-like structure within the β subunit has been suggested based on the similarity in hydropathy profiles between the I domain and part of the β subunit (7). Interestingly, this region of β has been reported to be critical for ligand binding and its regulation (reviewed in Ref. 8) (Fig. 1). The Asp-119 (β3) (9) and Asp-130 (β1) (10, 11) and the corresponding residues in β2 and β6 are critical for ligand binding (12, 13). A synthetic peptide of β3 (MDL5YSKDDLWSI, residues 118–131) has been shown to produce a ternary complex with cations and ligand (14). Also, the sequence DDLW (residues 126–129 of β3) was shown to be critical for interaction with the RGD sequence using a phage display system (15). A synthetic peptide of β3, DAPEGFDAMQT (residues 217–231 of β3), has been shown to bind to immobilized fibrinogen (Fg) (1) von Willebrand’s factor (vWF), and fibronectin (Fn) (16, 17). A synthetic peptide of β3, SVSRNMRGAEG (residues 211–221 of β3), has been reported to block binding of Fg to αIIbβ3 (18, 19). We identified a small region of β1 (residues 207–218, a regulatory epitope) that is recognized by both activating and inhibiting anti-β1 antibodies (20). These antibodies probably induce high or low affinity states, respectively, by changing the conformation of the β1 subunit through binding to the non-ligand binding site (20).

We and researchers at other laboratories have recently identified residues critical for ligand binding in the putative I-domain-like structure of β1 (6), β2 (21), and β3 (22). In β1, eight critical oxygenated residues are located in several separate predicted loop structures, which probably constitute multiple ligand/cation binding sites within the I-domain-like structure of the β subunit. These critical oxygenic residues are conserved among integrin β subunits, indicating that these residues are ubiquitously involved in ligand binding regardless of ligand and integrin species. We observed that a large predicted loop region (residues 176–199 of β1) is diverse among the β subunits (Fig. 1). Furthermore, a recent structural model (23) and our preliminary model (not shown) of the I-domain-like structure of β suggest that the sequence is also on the same side of the domain as residues critical for ligand binding. We hypothesized that the predicted loop (especially the disulfide-linked short sequences, e.g. residues 187–193 of β1) is involved in ligand specificity of integrins. αβ3 has been shown to recognize a wide variety of ligands, including Fn, Fg, vWF, and vitronectin (Vn); αβ3 is specific to Fn. We designed experiments, using αβ1 and αβ3 integrins, to determine whether a diverse sequence in the predicted loop (e.g. residues 176–199 in β1, residues 166–190 in β3) is involved in ligand specificity of integrins.

EXPERIMENTAL PROCEDURES

Antibodies—mAb 4B4 (to human β1) (24) was kindly provided by C. Morimoto (Dana-Farber Cancer Institute, Boston, MA); SA2 (to human β1) (25) by N. Kovach and J. Harlan (University of Washington, Seattle, WA); A1A5 (to human β1) (26) by M. Hemler (Dana-Farber Cancer Institute, Boston, MA); LM142 (to human αv), LM609 (to αβ3) (27), and P3G2 (to αβ3) (28) by D. Cheresh (Scripps); 15 (to human β3) (29)

The abbreviations used are: Fg, fibrinogen; CHO, Chinese hamster ovary; Fn, fibronectin; Vn, vitronectin; vWF, von Willebrand’s factor; mAb, monoclonal antibody; WT, wild type; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid.

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The diverse sequence within the putative I domain-like structure of the β subunit. This region of the β subunit contains eight predicted β-strands and five predicted α-helices (6). Eight critical oxygenated residues (Asp-130, Ser-132, Asn-224, Asp-226, Glu-229, Asp-233, Asp-267, and Asp-295 in β1) are located in several adjacent predicted loop structures (or at the boundary between a predicted α-helix and a β-strand), which probably constitute multiple ligand/cation binding sites within the I domain-like structure of the β subunit. Although most of the sequences of the predicted turn structures containing the critical oxygenated residues are conserved among integrin β subunits, a large predicted loop region (residues 176–199) is not conserved among the β subunits. Particularly, the sequence and size of the disulfide-linked short sequence (e.g. residues 187–193 in β1, boxed area in figure 1) is diverse. We hypothesized that the predicted loop region of the β subunit is involved in ligand binding specificity.

Affinity Chromatography—Cells were harvested with 3.5 mM EDTA in PBS and washed with PBS. Cells (about 5 x 10^6) were then surface-labeled with 125I by using IODO-GEN (Pierce) (37), washed three times with PBS, and solubilized in 1 ml of 100 mM octyl glucoside in 10 mM Tris-HCl, 0.15 mM NaCl, pH 7.4 (TBS), containing 2.5 mM MnCl2, 1 mM phenylmethylsulfonyl fluoride (Sigma) at 4 °C for 15 min. The insoluble materials were removed by centrifugation at 15,000 g for 10 min. The supernatant was incubated at 4 °C for 1 h with 200–500 µl of packed Fg-, Vn-, Fn 110-kDa fragment-, or GRGDS-Sepharose that had been equilibrated with TBS containing 2.5 mM MnCl2, 1 mM phenylmethylsulfonyl fluoride, 25 mM octyl glucoside (washing buffer). The unbound materials were washed with a 20 X column volume of washing buffer, and the bound materials were eluted with 20 mM EDTA instead of 1 M MnCl2 in washing buffer; and then 0.5-ml fractions were collected. Twenty-µl aliquots from each fraction were analyzed by SDS-polyacrylamide gel electrophoresis using 7% polyacrylamide gel followed by autoradiography.

Immunostaining—Glass coverslips (Fisher) were treated with 10% KOH in methanol for 1 h at room temperature, washed three times with distilled H2O, and stored in ethanol. Etched coverslips were then coated with 100 µl of pFneo; cells were then blocked with 10 µg/ml heat-denatured bovine serum albumin (Calbiochem) in PBS for 10 min at room temperature. For plating experiments, cells were washed and then detached with 2.5 mM EDTA/PBS. Detached cells were isolated, washed, resuspended in Dulbecco's modified Eagle's medium, and then replated on coated coverslips. Cells were allowed to attach and spread for 2 h. Prior to fixation, cells were chilled on ice for 5 min, washed with cold PBS, and then extracted with cold PIPES buffer (0.1 M PIPES, pH 6.8, 1 mM MnCl2, and 1 mM EDTA) containing 1% glyceraldehyde and 5% Nonidet P-40 (Polyscience) in cold PIPES buffer (0.1 M PIPES, pH 6.8, 1 mM MnCl2, and 1 mM EDTA). For example, cells were washed with cold PIPES buffer and then fixed with 3.7% formaldehyde in cold PIPES buffer (0.1 M PIPES, pH 7.4, 1 mM MgCl2, and 1 mM EGTA) containing 1% glycerol and 0.5% Nonidet P-40 for 1–2 min. Extracted cells were labeled with 125I by using IODO-GEN (Pierce) (37), washed three times with cold PIPES buffer, and then incubated with 125I-labeled antibody for 1 h at room temperature. The bound materials were then eluted with 20 mM EDTA instead of 1 M MnCl2 in washing buffer; and then 0.5-µl fractions were collected. Twenty-µl aliquots from each fraction were analyzed by SDS-polyacrylamide gel electrophoresis using 7% polyacrylamide gel followed by autoradiography.
either the anti-human β1 antibody P5D2 or the anti-human β3 antibody 15. Cells were immunostained for 1 h at 37 °C, washed, and then stained with a fluorescein isothiocyanate-conjugated sheep anti-mouse IgG secondary antibody (Molecular Probes) for 30 min at 37 °C; cells were also labeled with rhodamine phalloidin (Molecular Probes) to detect actin stress fibers. Stained cells were mounted in Fluoromount-G (Fisher) and photographed using a Nikon Diaphot inverted microscope.

Other Methods—Site-directed mutagenesis of the β1 and β3 cDNA in a pBβ1-1 vector was carried out using unique restriction site elimination (38). The presence of mutations was confirmed by DNA sequencing. Immunoprecipitation was carried out as described previously (20).

RESULTS

Swapping the CTSEQNC Sequence of β1 (residues 187–193) with the Corresponding CYDMKTTC Sequence of β3 Induces Adhesion of αvβ1 to Fg and vWF—To determine whether a diverse sequence in the predicted loop (residues 176–199 in β1 and residues 166–190 in β3) is involved in ligand specificity of integrins, we replaced the CTSEQNC sequence of β1 with the corresponding CYDMKTTC sequence of β3 by site-directed mutagenesis. The CYDMKTTC sequence of β3 has been reported to be disulfide-linked (39). The resulting mutant β1-3-1, wild-type β1, or wild-type β3 cDNA constructs were transfected into either parental CHO cells or CHO cells expressing wild-type human αv (αvCHO). Parent CHO cells have been reported to express endogenous hamster αv (40) but not β3 (41). Consistent with these findings, we found that CHO cells express αvβ5 using mAb P3G2 (data not shown). The cloned cells expressing WT or mutant β1 in association with exogenous human αv were designated αvβ1-, αvβ1-3-1-, αvβ3-CHO cells, and those with only endogenous hamster αv were designated as β1-, β1-3-1-, β3-CHO cells.

αvβ3 recognizes multiple ligands, including Fn, Fg, vWF, and

![Image](image-url)
Vn; αvβ1 is specific to Fn on CHO cells. Therefore, we tested the ligand specificity of the β1–3-1 mutant. As shown in Fig. 2A, we found that cells expressing αvβ3 or αvβ1–3–1, but not αvβ1, adhered to both Fg and vWF. Adhesion of the αvβ1–3–1 but not αvβ3–CHO cells was blocked by the inhibitory anti-human β1 mAb P5D2 (for αvβ1 and αvβ1–3–1 CHO cells) or the anti-human β3 mAb 15 (for αvβ3–CHO cells) and fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody. αvβ1–CHO cells did not attach to a Fg-coated glass plate.

Binding Specificity of αvβ1–3–1 to Fg, Vn, and Fn 110-kDa Fragment Immobilized to Sepharose—The specificity of the interaction between αvβ1–3–1 and ligands was further analyzed by affinity chromatography. Lysates from surface 125I-labeled αvβ1–3–1–CHO cells (as well as control αvβ1- and αvβ3–CHO cells) were incubated with immobilized Fg or Fn 110-kDa fragments, and bound materials were eluted with EDTA. As shown in Fig. 3A, bands corresponding to αv and β1 in size were eluted from Fg–Sepharose using a lysate of αvβ1–3–1 CHO cells, while bands corresponding to human αv and β3 were eluted from Fg–Sepharose with a lysate of αvβ3–CHO cells. Immunoprecipitation of the eluate from αvβ1–3–1 cells using anti-β1 mAb A1A5 (Fig. 3C, lane 5) and anti-αv mAb LM142 (Fig. 3D, lane 5) confirmed that these two bands are human αv and β1 (β3–1–3). In contrast, very low levels of αv and β1 were detected in the Fg–Sepharose eluate with lysate of αvβ1–CHO cells. These results suggest that αvβ1–3–1 exhibits a much higher affinity for Fg than αvβ1. Similar results were obtained with Vn–Sepharose (data not shown), suggesting that αvβ1–3–1 shows a much higher affinity to Vn as well. In experiments done in parallel, we have detected bands corresponding to αvβ1, αvβ1–3–1, and αvβ3 in the eluate from Fn 110-kDa fragment–Sepharose with lysates from αvβ1, αvβ1–3–1, and αvβ3–CHO cells, respectively (Fig. 3B). Immunoprecipitation confirmed that the major β subunits in the eluates are β1, β3–1–3, and β3, respectively (Fig. 3C). These results suggest that the αvβ1–3–1 mutant, like αvβ3, binds to Fn, Vn, and Fn 110-kDa fragments in a solubilized form.

The αvβ1–3–1 Mutant Is Recruited to Focal Contacts and Transduces Signals on Adhesion to Fg and Vn—Next we determined if the altered ligand specificity of the αvβ1–3–1 chimeric affected intracellular signaling. Cells were plated on Fn, Vn, or Fg, and localization of the human integrin was determined by immunostaining with anti-human β1 (αvβ1 and αvβ1–3–1) or anti-human β3 (αvβ3). While all three receptors localized to focal adhesions in cells plated on Fn (Fig. 4, A, C, and F), only β1–3–1 and β3 localized to focal adhesions in cells on Vn; αvβ1–CHO cells did attach and spread on Vn due to endogenous αvβ5. However, αvβ1 exhibited a diffuse staining pattern. This result is consistent with the binding data and indicates that the αvβ1–3–1 chimera is able to generate intracellular signals. In addition, we found that the αvβ1–3–1 chimera, like αvβ3, induced cell spreading and focal adhesion formation in cells plated on Fg; αvβ1 cells did not adhere to Fg. Similar results were obtained with the β1–, β1–3–1-, and β3–CHO cells that express lower levels of the transfected integrins (data not shown). These results indicate that the αvβ1–3–1 chimera is a functional receptor and has the same signaling properties as αvβ3.

The Reciprocal β3–1–3 Swapping Mutation Blocks Binding of αvβ3 to Fg, vWF, and Vn and to LM609, a Function-blocking Anti-αvβ3 mAb—To determine whether the reciprocal swapping mutation has any effect on the ligand specificity of αvβ3, we replaced the CYDMKTC sequence of β3 (residues 177–184) with the corresponding CTSEQNC sequence of β1 (the β3–1 mutation). The resulting mutant β3–1–3 and WT β3 cDNA constructs were transfected into αvβ3–CHO cells, and cells stably expressing αvβ3–1–3 or αvβ3 were cloned by sorting (αvβ3–1–3–CHO and αvβ3–CHO cells, respectively). The levels of αv and β3 expression were comparable in clonal WT αvβ3–CHO and αvβ1–3–3–CHO cells used. αvβ3–1–3–CHO cells showed significantly lower adhesion activity than αvβ3–CHO cells to both Fg and vWF. αvβ3–1–3–CHO cells required higher ligand concentrations for adhesion than WT αvβ3–CHO cells (Fig. 5, A and B). In addition, solubilized αvβ3–1–3 did not bind to either Fg or Vn immobilized to Sepharose, although solubilized WT αvβ3 did (Fig. 5C). These results suggest that the β3–1–3 mutation significantly reduces binding of αvβ3 to Fg, Vn, and vWF. Although we observed that αvβ3–1–3 mutant binds to Fn 110-kDa fragments and to the GRGDSP peptide on affinity chromatography (data not shown), we could not determine whether the β3–1–3 mutation changes the binding affinity of αvβ3 to Fn 110-kDa fragment or the GRGDSP peptide.
verse effect on the other receptor functions (e.g. α-β association and signal transduction). In reciprocal experiments, swapping a disulfide-linked CYMDKTTG sequence of β3 with the corresponding CTSEQNC sequence of β1 blocks the binding function of αβ3 to Fg, vWf, and Vn. Taken together, the present study suggests that a small disulfide-linked CYMDKTTG sequence of β3 (and the CTSEQNC sequence of β1 as well) defines a novel site of integrin β critical for ligand specificity. Sequence diversity among β subunits and localization within an I domain-like structure of β, close to putative ligand binding sites (see Introduction) is consistent with the proposed function of the sequence. In a preliminary study, we introduced mutations into the corresponding predicted loop of the β2 subunit. We found that these mutations showed profound effects on the ligand binding function of αLβ2 integrin, 2 indicating that the diverse predicted loops of the β subunits are ubiquitously involved in the regulation of ligand binding functions.

Mechanisms by which the disulfide-linked sequences in a predicted loop within the I domain-like structure of the β subunits define ligand specificity of integrins have yet to be studied. In preliminary studies, we did not obtain evidence that the β1–3–1 mutation induces constitutive activation of β1 integrins or induces drastic conformational changes. We determined the reactivity of the β1–3–1 mutant on conformation and binding function of αLβ2 integrin, 2 indicating that the diverse predicted loops of the β subunits are ubiquitously involved in the regulation of ligand binding functions.

(because of the presence of other fibronectin receptors, endogenous αβ3 and αδβ3).

The immunoprecipitation of whole lysate of αβ3–1–3–CHO cells using anti-α and anti-β mAbs showed that anti-α and anti-β co-precipitated β3–1–3 and α subunits, respectively, suggesting that the β3–1–3 mutation does not affect the α-β association. However, the αβ3–1–3 mutant did not react with LM609, a function-blocking anti-αβ3 mAb, upon immunoprecipitation (Fig. 6) and flow cytometric analysis (data not shown), suggesting that the β3–1–3 mutation destroyed the LM609 epitope and that the CYMDKTTG sequence of β3 is closely located to ligand binding sites of αβ3.

**DISCUSSION**

We established that swapping the CTSEQNC sequence of β1 with the corresponding CYMDKTTG sequence of β3 induces significant changes in ligand specificity of αβ1. The β1–3–1 mutation markedly increases affinity of αβ1 to Fg, vWf, and Vn (a gain-of-function effect). Since the αβ1–3–1 mutant is functional in cultured cells and transduces signals on adhesion to the ligands, the swapping did not induce a detectable ad-

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1. T. Kamata and Y. Takada, unpublished results.

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![Image](97x400 to 258x729)

**Fig. 5.** The β3–1–3 mutation blocks binding of αβ3 to Fg, vWf, and Vn. Adhesion of αβ3–1–3–CHO cells to Fg (A) and vWf (B) is shown. Wells of 96-well microtiter plates were coated with varying concentrations of Fg and vWf. Cells (105 cells/well) in 100 µl of Dulbecco’s modified Eagle’s medium were added to the wells and incubated at 37 °C for 1 h. After gently rinsing the wells three times with PBS to remove unbound cells, bound cells were quantified. Mean fluorescence intensities are 246 for human αv and 214 for human β3 in WT αβ3–CHO cells and 331 for human αv and 262 for human β3 in αβ3–1–3–CHO cells. C, affinity chromatography of αβ3–1–3 and αβ3–CHO cells on Vn and Fg. Lysates of surface 125I-labeled cells were incubated with Vn-Sepharose or Fg-Sepharose that had been equilibrated with buffer containing 2.5 mM MnCl2. The bound materials were eluted with 20 mM EDTA. Twenty-µl aliquots from the first four 0.5-ml fractions were analyzed by SDS-polyacrylamide gel electrophoresis using 7% polyacrylamide gel under nonreduced conditions.

![Image](384x608 to 488x729)

**Fig. 6.** The β3–1–3 mutation blocks binding of αβ3 to LM609, a function-blocking anti-αβ3 antibody. Immunoprecipitation of αβ3 and αβ3–1–3 is shown, LM142 (to human αv), polyclonal anti-αv cytoplasmic peptide antibody, 15 (to human β3), LM609 (to αβ3).
FIG. 7. Positions of residues critical for ligand specificity in a hypothetical model of the I domain-like structure of β3. This hypothetical model was taken from Ref. 23 and modified. Arrows indicate β-sheets, and columns indicate α-helices. Closed circles show residues critical for ligand binding in β1 (6). In this model, the diverse disulfide-linked sequence critical for ligand specificity (residues 187–193 of β1 and 177–184 of β3) is located in the predicted loop, surrounded by conserved oxygenated residues critical for ligand binding (e.g. Asp-130 in β1) in the upper face of the I domain-like structure of β3. The upper face of this domain is predicted as a ligand binding site, based on the homology to the I domains of αM and αL (23). The small region that is recognized by activating or inhibiting antibodies (residues 207–218, a regulatory epitope) (20) is located in a predicted loop on the other side of the domain (a non-ligand binding site).

(7), and this model is similar to our preliminary model (not shown). All of the residues critical for ligand binding (e.g. Asp-130 and Glu-229 of β1) (6, 10) are located in the upper face of the model (predicted as the ligand binding site). Also, the regulatory epitope (residues 207–218 of β1) (20), which is recognized by both activating and inhibiting anti-β1 mAbs, is located in the non-ligand binding site (in the lower face) of the domain. Interestingly, a diverse sequence in the predicted loop (e.g. residues 176–199 in β1, residues 186–190 in β3), which is involved in ligand specificity of integrins in the present study, is located in the upper face of the domain in this model. The finding that the β3–1–3 mutation blocked binding of the function-blocking anti-αβ3 antibody LM609 supports the idea that the predicted loop structure is close to the ligand binding site of αβ3. Taken together, the present and previous mutagenesis data strongly support this model. Recently, Collins Tozer et al. (22) published an interesting atomic model of the putative I domain of β3, which is based on the crystal structure of the αM I domain (7). However, our mutagenesis data do not fit in very well with their model, since 1) the sequence CYDMKTTC of β3, which is critically involved in ligand binding to αβ3, is not close to the MIDAS site (apparently in a non-ligand binding site) in their model, and 2) although Thr-197 of β3 is located in the MIDAS site of β3 in this model, the corresponding residue of β1 (Thr-206) is very close to the regulatory epitope. This epitope is probably located in a non-ligand binding site of β1 because 1) binding of some mAbs actually activates, instead of inactivating, the β1 integrins, and 2) this epitope has recently been shown to be an allosteric effector site of β1 (46), since the binding of an inhibitory anti-β1 mAb 13 to the regulatory epitope is also dramatically attenuated by ligands (Fn fragments or the GRGDS peptide). Further biochemical and structural characterization of this region of the β subunit may be required to substantiate these models. αβ3 has been shown to be involved in the progression of melanoma and induction of neovascularization by tumor cells. αβ3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels (47, 48). We identified a critical region for ligand binding and specificity of integrins using a gain of function mutant of the β subunit. The predicted loop sequence of the integrin β3 subunit is a new potential target for designing inhibitors of ligand binding functions of αβ3.

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