α3β1 Adhesion to Laminin-5 and Invasin: Critical and Differential Role of Integrin Residues Clustered at the Boundary between α3 N-Terminal Repeats 2 and 3[†]

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ABSTRACT: Integrin/ligand interaction is a therapeutic target for many diseases. We previously reported that residues critical for ligand binding are clustered in N-terminal repeat 3 (in the predicted 2-3 loop) of α4, α5 and αIIb. Here we have localized residues critical for ligand binding in the α3 subunit of integrin α3β1 with distinct ligand specificity (laminin-5). We identified an α3 epitope common to several function-blocking anti-α3 antibodies at the boundary between repeats 1 and 2 (residues 75−80). We found that swapping the predicted 4-1 loop (residues 153−165) at the boundary between repeats 2 and 3 with the corresponding α4 sequence and mutating Thr-162 and Gly-163 residues in this predicted loop block laminin-5 binding. Thr-162 and Gly-163 and the antibody epitope are separated in the primary structure; however, they are close to each other in the proposed β-propeller model. Mutating residues recently reported to block (Tyr-186 and Trp-188) or enhance (Asp-122) laminin-5 binding to α3β1 [Krukonis, E. S., Dersch, P., Eble, J. A., and Isberg, R. R.(1998) *J. Biol. Chem. 273*, 31837−31843] did not affect laminin-5 binding under the assay conditions used. Thr-162 and Gly-163 are not critical for adhesion to invasin, indicating that laminin-5 and invasin may use different recognition mechanisms, and that mutation of Thr-162 and Gly-163 does not drastically affect the integrity of α3β1. These results suggest that residues critical for ligand binding may be similarly (but not identically) located in repeat 3 of the α subunit regardless of ligand specificity.

Integrins are a supergene family of cell adhesion receptors that mediate cell/extracellular matrix interactions through multiple ligands (*1*−*3*). Integrins are involved in the pathogenesis of many diseases in humans and in animal models. Integrin α3β1 is a receptor for laminin-5 (*4*). Inhibition of keratinocyte adhesion via α3β1/laminin-5 interaction induces epidermal differentiation (*5*). A defect in α3 expression in transgenic mice has been reported to induce abnormal development of the lung and kidney (*6*), indicating that this integrin may play a crucial role in basement membrane organization and branching morphogenesis (*6*). DiPersio et al. demonstrated the requirement for α3β1 in basement membrane organization (*7*). Laminin-5, a unique laminin variant that is essential for epidermal adhesion, is composed of three nonidentical subunits, α3, β3, and γ2. Genetic defects in laminin-5 components have been reported in patients suffering from hereditary junctional epidermolysis bullosa, a lethal congenital skin disease (*8*−*11*). Lampe et al. (*12*) recently reported that adhesion of epithelial cells to laminin-5 in the basement membrane via α3β1 promotes gap junctional intercellular communication that integrates the individual motile cells into a synchronized colony. Also, cleavage of laminin-5 by matrix metalloprotease-2 and the resulting exposure of the cryptic site have been reported to promote α3β1-mediated migration of tumor cells, indicating that the α3β1/laminin-5 interaction is a target for modulation of tumor cell invasion and tissue remodeling (*13*). These reports indicate that elucidation of the mechanisms of α3β1/laminin-5 interaction is critical for understanding the roles of α3β1 in biological and pathological processes and for designing potential therapeutics. The ligand binding mechanisms of non-I-domain integrins have been extensively studied (reviewed in refs *14*−*16*). However, the role of the non-I-domain α subunit has not been established, and our understanding of integrin/laminin-5 interaction is limited. Krukonis et al. (*17*) reported that mutation of Tyr-186 and Trp-188 to Ala in α3 blocks α3β1 adhesion to laminin-5, and that mutation of Asp-122 to Ala in α3 increases the efficiency of α3β1 adhesion to laminin-5.

The integrin α subunit has 7 repeated sequences of 60−70 residues each in its amino terminal. In the present study, we used interspecies α3 chimeras to identify an epitope common to several function-blocking anti-α3 antibodies at

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the boundary between α3 N-terminal repeats 1 and 2 (residues 75−80). We used swapping the predicted loops and alanine-scanning mutagenesis to identify critical region/residues for laminin-5 binding at the boundary between repeats 2 and 3 (Thr-162 and Gly-163). The two epitopes (residues 75−80 and Thr-162/Gly-163) are separated in the primary structure, but are close to each other in the proposed β-propeller model (*18*), consistent with this model. These results suggest that Thr-162 and Gly-163 at the boundary between repeats 2 and 3 of α3 are critical for adhesion to laminin-5 or its regulation. These residues are not critical for α3β1 binding to invasin. We did not detect any significant effects of mutation of Asp-122, Tyr-186, and Trp-188 to Ala (*17*) on adhesion to laminin-5 under the assay conditions used. These results suggest that residues critical for ligand binding may be similarly located in repeat 3 of the α subunit regardless of ligand and integrin species.

EXPERIMENTAL PROCEDURES

Antibodies. (A) Anti-Human α3 mAbs.[1] P1B5 and P1F2 (*19*) were provided by Drs. E. Wayner and W. G. Carter (Fred Hutchinson Cancer Institute, Seattle, WA). SM-S1, SM-S2, SM-T1, and SM-T7 were prepared as described in ref *20*. ASC-1, ASC-5, ASC-6, and ASC-10 were obtained from the VIth International Leukocyte Differentiation Antigen Workshop. 7A8 was generated as described (*21*).

(B) Other mAbs. The activating anti-human β1 mAb 8A2 was developed as described in ref *22*. KH/72 (anti-α5, unpublished) is a kind gift from K. Miyake (Saga Medical School, Saga, Japan). Anti laminin-5 mAbs C2-5 and C2-9 (*23*) were provided by W. G. Carter.

Construction of α3 Chimeras and Mutants. Sequences in α3 were swapped with the corresponding sequences of α4 using the primer extension polymerase chain reaction method (*24*). Site-directed mutagenesis was carried out by unique site elimination using a double-stranded vector (*25*). The mutations were confirmed by DNA sequencing. Hamster α3 (*26*) and human α3 (*27, 28*) cDNAs were obtained as described in the cited references. cDNAs for interspecies chimeric α3 were prepared by fusing hamster α3 cDNA and human α3 cDNA using the following common restriction sites: a *Pst*I site for the r102/h and h102/r α3 chimeras; an *Nde*I site for the r245/h and h245/r α3 chimeras; and an *Eco*RI site for the h766/r α3 chimera. Wild-type (wt) and mutant cDNAs were subcloned into a pBJ-1 expression vector (*29, 30*).

Transfection and Selection of Cells Expressing α3 Mutants. CHO-K1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum at 37 °C in a 6% CO_2 incubator. Ten micrograms of the cDNA construct was transfected into CHO-K1 cells (8×10^6 cells) by electroporation together with 1 μg of pFneo DNA with a neomycin-resistance gene. Transfected cells were maintained for 2 days in the above medium, and then were transferred to the same medium supplemented with 700 μg/mL G418 (Gibco). After 10−14 days, the resulting colonies were harvested. Usually, 30−50% of the harvested cells express α3 as measured by flow cytometric analysis. Transfection of K562 cells was carried out as described, except that RPMI 1640 medium and 1 mg/mL G418 were used.

Adhesion Assay. Laminin-5 ECM was prepared according to ref *31*. Briefly, HaCaT human keratinocyte cells (provided by Dr. N. E. Fusenig, German Cancer Research Center, Heidelberg, Germany) or A431 human epidermal carcinoma cells were grown overnight in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum in 96-well tissue culture plates. The subconfluent cell layer was treated with 0.05% trypsin and 1 mM EDTA in PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4) for 5−10 min to remove cells. The wells containing laminin-5 ECM were rinsed with PBS containing soybean trypsin inhibitor (0.1 mg/mL) and bovine serum albumin (1 mg/mL) and used for adhesion assays. K562 cells expressing wt or mutant α3 were plated on wells (10^5 cells per well) and incubated for 1 h at 37 °C in RPMI 1640 medium in the presence or absence of activating anti-β1 mAb 8A2 (ascites at 500× dilution). After rinsing with PBS to remove unbound cells, bound cells were quantified using endogenous phosphatase assay (*32*). We obtained essentially identical results with either HaCaT cells or A431 cells.

MBP-Inv479 (invasin) was prepared using pJL309 plasmid in *E. coli* MC1000 that was provided by R. Isberg (Tufts University, Boston, MA) (*33*). Wells of a 96-well microtiter plate (Immulon-2) were incubated with invasin in 100 μL of PBS (0.1 μg/mL) overnight at 4 °C. K562 cells expressing wt or mutant α3 were plated on wells (10^5 cells per well) and incubated for 1 h at 37 °C in RPMI 1640 medium in the presence of function-blocking anti-α5 mAb KH72 (ascites at 500× dilution) to block α5β1 on K562, another receptor for invasin.

Other Methods. Flow cytometry was performed as previously described (*34*).

RESULTS

α3β1-Mediated Cell Adhesion to Laminin-5 ECM. We used K562 cells expressing wt recombinant human α3 (α3-K562 cells) to determine α3β1-mediated cell adhesion to laminin-5 ECM, which is secreted by keratinocytes (*4, 35, 36*). The wt α3-K562 cells adhered to laminin-5 ECM in the presence of 8A2, an activating anti-β1 mAb, but the parent K562 cells did not. Wt α3-K562 adhesion without 8A2 was typically less than 30% of the level shown with 8A2. Adhesion of wt α3-K562 was blocked by EDTA (data not shown) and anti-α3 mAbs (Figure 2). Adhesion of α3-K562 to laminin-5 ECM was effectively blocked by the inhibitory anti-laminin-5 mAb C2-9 (*31*), but not by the noninhibitory anti-laminin-5 mAb C2-5. Parent K562 cells express endogenous α5β1 (fibronectin receptor), and 8A2 increases binding of fibronectin to K562 cells (*37*). Adhesion of parent K562 cells to laminin-5 ECM is not stimulated by 8A2, and therefore is not α5β1- or fibronectin-dependent. Consequently, under the assay conditions used, α3-K562 interaction with laminin-5 ECM is specific to the α3β1 integrin, and laminin-5 is a predominant ligand for α3β1. We added mAb 8A2 to the adhesion assay mixture in all of the experiments that used K562 cells expressing α3 mutants in order to highly activate α3β1.

Identification of an Epitope That Is Recognized by Function-Blocking Anti-α3 Antibodies. Figure 2 shows that eight

[1] Abbreviations: ECM, extracellular matrix; mAb, monoclonal antibody; PBS, phosphate-buffered saline; wt, wild type.

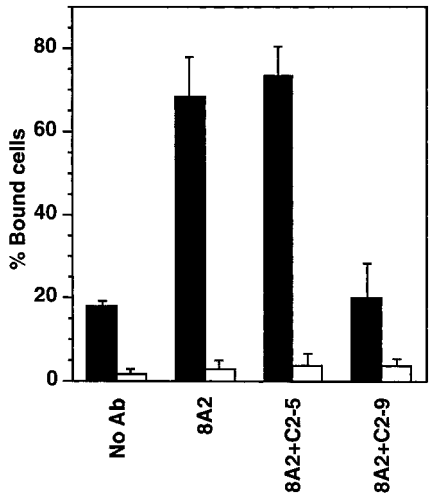

FIGURE 1: Adhesion of α3-K562 cells to laminin-5. Parent K562 (open bars) or wt α3-K562 (solid bars) cells (10^5 cells per well) were incubated with laminin-5 ECM for 1 h at 37 °C in RPMI 1640 medium. The activating anti-β1 mAb 8A2 (ascites at 500× dilution) and/or the anti-laminin-5 mAbs (supernatant at 4× dilution) were included. The anti-laminin-5 mAb C2-9 blocks cell adhesion to laminin-5 (*23*). The anti-laminin-5 mAb C2-5 does not block cell adhesion to laminin-5. After rinsing the wells to remove floating cells, bound cells were quantified using the endogenous phosphatase assay (*32*). The expression profile of wt α3 on K562 cells is shown in Figure 5. Data are expressed as means ± SD of triplicate experiments. The data suggest that adhesion of K562 cells to laminin-5 ECM is mediated by α3β1, is increased by activation by 8A2, and is effectively blocked by function-blocking anti-laminin-5 mAb.

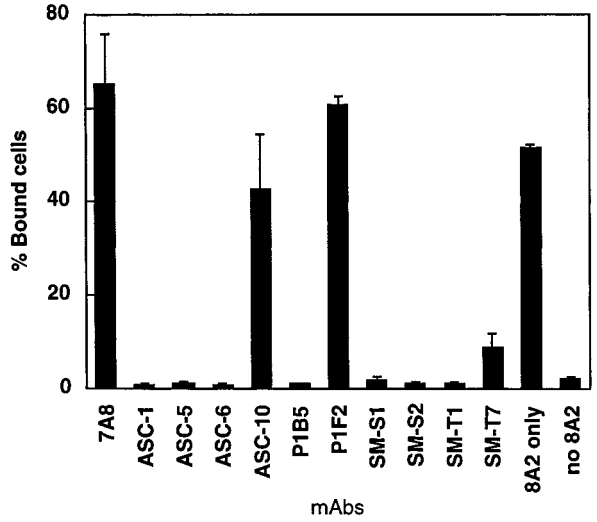

FIGURE 2: Effects of anti-α3 mAbs on adhesion of α3-K562 cells to laminin-5. Wt α3-K562 cells were incubated with laminin-5 as described above in the presence of 8A2 and various anti-α3 mAbs. Ascites (7A8) were used at 1000× dilution. Purified protein solutions (ASC-1 to ASC-10) were used at 1−2 μg/mL. Culture supernatants (P1B5, P1F2, and SM series) were used at 5× dilution. We confirmed that the saturating concentrations of mAbs were used. Data are expressed as means ± SD of triplicate experiments. The data suggest that all of the mAbs except for 7A8, ASC-10, and P1F2 strongly inhibit adhesion to laminin-5 under the conditions used.

of the anti-α3 mAbs tested strongly block adhesion of α3-K562 cells to laminin-5 ECM, but three of the anti-α3 mAbs (P1F2, ASC-10, and 7A8) do not block adhesion under the conditions used. To localize regions of α3 that are critical for ligand binding, we first localized epitopes for function-blocking antibodies using inter-species α3 chimeras. We developed chimeric cDNA constructs between human and hamster α3 (Figure 3a) and transfected them into CHO cells. Reactivity of wt and chimeric α3 to anti-α3 antibodies was then examined by flow cytometric analysis of cells stably or transiently expressing α3 chimeras (Table 1). All antibodies tested recognized the h766/r α3 chimera (in which the 766 N-terminal residues were derived from human α3 and the rest were from hamster α3). 7A8 and ASC-10 recognized the r245/h, r102/h, and h766/r α3 chimeras, but not the h245/r or h102/r α3 chimeras, indicating that nonfunctional 7A8 and ASC-10 recognize residues 245−766 of α3. All of the other antibodies recognized the h245/r, h102/r, and h766/r α3 chimeras, but not the r245/h and r102/h α3 chimeras, suggesting that all of the function-blocking antibodies and one nonfunctional antibody (P1F2) recognize a region spanning residues 1−102 of α3.

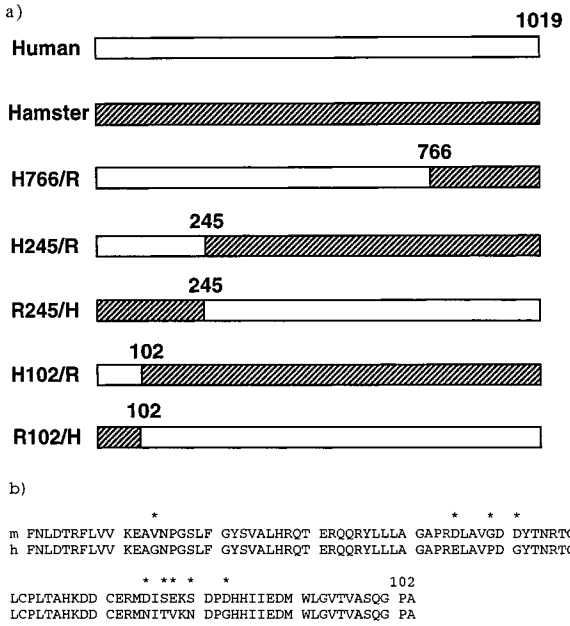

FIGURE 3: Interspecies α3 chimeras used for epitope mapping. (a) cDNAs for interspecies chimeras were prepared by fusing hamster α3 cDNA to human α3 cDNA using common restriction sites: a *Pst*I site for the r102/h α3 and h102/r α3 chimeras; an *Nde*I site for the r245/h and h245/r α3 chimeras; and an *Eco*RI site for the h766/r α3 chimera. (b) The 102 N-terminal residues from mouse and human α3 were aligned. An asterisk represents the difference between the two sequences. Human-to-mouse mutations were introduced alone (the G14V mutation) or in groups (the E45D/P49G/G51D and N75D/T77S/V78E/N80S mutations).

To further define the epitope for function-blocking antibodies, we introduced a human-to-mouse mutation into the epitope region (residues 1−102) either alone (the G14V mutation) or in groups (the E45D/P49G/G51D and the N75D/T77S/V78E/N81S mutations) (Figure 3b). The mutants were transiently expressed in CHO cells, and the reactivity of the mutant α3 to anti-α3 antibodies was determined by flow cytometric analysis (Table 2). The N75D/T77S/V78E/N80S mutation blocked all of the function-blocking mAbs tested, and the E45D/P49G/G51D mutation blocked several function-blocking mAbs (SM-S1, SM-S2, and SM-T1) and P1F2, a non-function-blocking mAb. The G14V mutation did not affect the binding of the mAbs tested. These results suggest that all of the function-blocking mAbs recognize the NITVKN$^{75-80}$ sequence at the boundary between repeats 1

Table 1: Reactivity of Human/Hamster Chimeric α3 to Anti-α3 mAbs[a]

	CHO	human wt α3	h766/r α3	h245/r α3	r245/h α3	h102/r α3	r102/h α3
mouse IgG	3.0	1.4	3.5	0.7	1.2	0.7	1.1
7A8	1.5	94.6*	33.5*	2.1	18.1*	0.6	21.9*
ASC-1	5.3	98.7*	24.2*	24.8*	1.0	15.8*	1.7
ASC-5	5.4	96.7*	17.3*	18.5*	1.4	14.4*	1.5
ASC-6	5.0	95.0*	16.8*	14.4*	1.5	11.3*	1.8
ASC-10	5.8	99.3*	31.4*	3.2	26.0*	0	27.7*
P1B5	2.3	91.8*	42.0*	42.6*	1.2	27.3*	0.7
P1F2	2.3	93.3*	42.5*	38.4*	1.1	32.8*	0.8
SM-S1	2.1	83.6*	38.7*	36.9*	0.9	32.8*	0.9
SM-S2	2.1	76.5*	41.9*	42.5*	0.7	26.9*	1.0
SM-T1	2.0	87.0*	48.2*	44.3*	1.2	35.8*	1.2
SM-T7	2.0	62.4*	29.4*	26.9*	0.8	28.5*	0.8

[a] Numbers in the table represent % positive cells. (*) represents positive reactivity. The h102/r α3 chimera was transiently expressed and tested for reactivity to mAbs. Other experiments were done with stably expressing cells. Human wt α3-CHO is clonal. The levels of positive signals with anti-human α3 mAbs may vary depending on the efficiency of transfection, the affinity of antibody binding, and the stability of mutants. Although the level of % positive cells is lower in the h102/r mutant than those of other mutants, there is a clear difference in negative reactivity (e.g., mouse IgG) and positive reactivity (e.g., ASC-6).

Table 2: Reactivity of Anti-α3 mAbs to the Human-to-Mouse α3 Mutants[a]

	parent CHO	G14V	E45D/P49G/ G51D	N75D/T77S/ V78E/N80S	wild type
mouse IgG	0.52	2.5	3.5	4.4	1.9
ASC-1	0.35	81.6*	88.0*	4.6	80.4*
ASC-5	0.55	78.2*	84.5*	3.7	76.4*
ASC-6	0.43	70.8*	77.1*	3.2	68.6*
P1B5	0.4	80.1*	87.7*	3.2	76.2*
P1F2#	0.32	75*	3.0	85.8*	66.6*
SM-S1	0.45	79.1*	3.8	4.1	72.1*
SM-S2	0.38	80.5*	2.8	4.2	76.7*
SM-T1	0.33	81*	2.9	3.8	76.1*
SM-T7	0.36	61.8*	67.6*	3.4	62.0*

[a] Wild-type and mutant α3 were transiently expressed on CHO cells. Reactivity to mAbs was determined using flow cytometry. Numbers in the table represent % positive cells. (*) positive reactivity; (#) non-function-blocking.

and 2, and several function-blocking mAbs recognize the ELAVPDG$^{45-51}$ sequence in repeat 1. Since P1F2 recognizes the ELAVPDG$^{45-51}$ sequence, but does not show function-blocking activity, the NITVKN$^{75-80}$ sequence may be related to the function-blocking activities of mAbs. These mutants were expressed on K562 cells and tested for their ability to adhere to laminin-5 ECM. These mutations did not affect binding to laminin-5 ECM.

A Predicted Loop Critical for Adhesion to Laminin-5. To localize regions of α3 that are critical for adhesion to laminin-5, we used domain-swapping mutagenesis. Predicted loops are located on the surface of the molecule and are primary candidates for ligand binding sites (*38*). Therefore, we expected that swapping the predicted loops with the corresponding regions of other α subunits with different ligand binding specificities would lead to the identification of regions of α3 that are critical for ligand binding. We replaced several predicted loop regions of α3 within or close to the epitope region spanning residues 75−81 (residues 41−57, 62−71, 108−130, 153−165, and 181−190) with the corresponding sequences of α4 or α5 according to the secondary structure prediction in refs *18* and *39* (Figure 4). The mutant α3 cDNAs were then transfected into K562 cells together with a neomycin-resistant gene. After selection with G418, we detected stable expression of two of the swapping mutants, designated α3(153−165/α4) and α3(41−57/α5). Flow cytometric analysis did not indicate expression of any other chimeric α3. Cells stably expressing the α3(153−165/α4) or α3(41−57/α5) mutants were cloned by cell sorting to obtain high expressors. Figure 5 shows that the K562 cells almost homogeneously express the α3(153−165/α4) mutant. The α3(41−57/α5) mutant showed adhesion to laminin-5 ECM at a level comparable to that of wt α3 (Figure 6). Interestingly, the α3(153−165/α4) mutant did not show any significant adhesion to laminin-5 ECM. In contrast, cells expressing mutants at a level comparable with or lower than that of the α3(153−165/α4) mutant (e.g., the S18A mutant) showed significant adhesion to laminin-5 ECM, suggesting that the expression of α3(153−165/α4) may be high enough to support adhesion of K562 cells. The data indicate that the predicted loop sequence CNSNTDYLETGMC$^{153-165}$ of α3 may be critically involved in α3β1/laminin-5 interaction. All of the function-blocking and non-function-blocking anti-α3 antibodies in Table 1 bind to the α3(153−165/α4) mutant (data not shown). Therefore, this mutation is not likely to induce drastic structural changes in α3β1. It is still possible that the mutation indirectly blocks binding to laminin-5 ECM by blocking signal transduction through α3β1. We determined whether the mutation blocks spreading of CHO cells with mAb P1B5 as a ligand. We found that P1B5 supports adhesion and spreading of wild-type and mutant α3, but does not support adhesion of parent CHO cells. These results suggest that the 153−165/α4 mutation does not affect signal transduction through α3β1.

Residues Critical for Adhesion to Laminin-5 in the Predicted Loop Sequence CNSNTDYLETGMC$^{153-165}$. It is possible that swapping large domains may indirectly affect ligand binding function through conformational changes. To minimize the level of mutation, we introduced multiple point mutations within and around the predicted loop region that is critical for ligand binding (residues 153−165). We used alanine-scanning mutagenesis, which yields high-resolution epitope mapping (*40*). The α3 mutants were expressed on K562 cells, and cell adhesion to laminin-5 was examined. We found that the mutations T162A (Thr-162 to Ala), T162F (the corresponding α4 residue is F), and G163A blocked adhesion to laminin-5 ECM (Figure 7), but mutations of other residues in this region did not affect adhesion to laminin-5

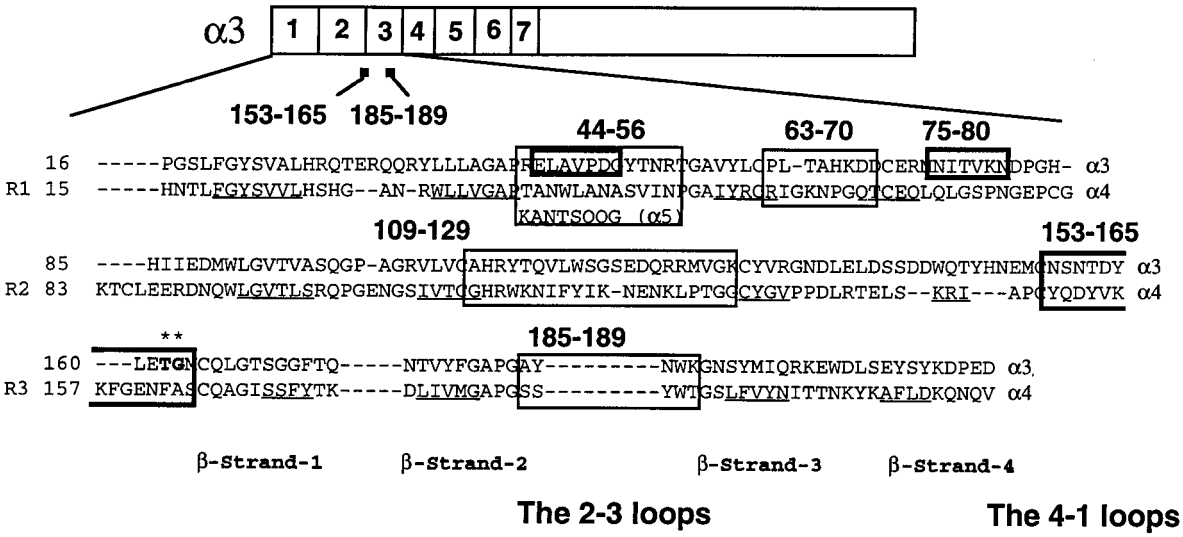

FIGURE 4: Regions of α3 selected for swapping in this study. Integrin α subunits have 7 repeats of about 60 amino acid residues each at their N-terminals. We chose predicted loop structures of α3 that are either close to the epitope region for function-blocking anti-α3 antibodies (residues 75−80) or within the previously identified putative ligand binding sites of α4 (residues 108−268) (*52*) for swapping with the corresponding residues of α4 (boxed regions). R1−R3 represent repeats 1−3 (*18*). The predicted *β*-strands of α4 are underlined. In the *β*-propeller model, residues 153−165 of α3 represent the 4-1 loops (the loops between *β*-strands 4 and 1). Both the 2-3 and the 4-1 loops are in the upper face (predicted ligand binding site) of the proposed *β*-propeller model (*18*).

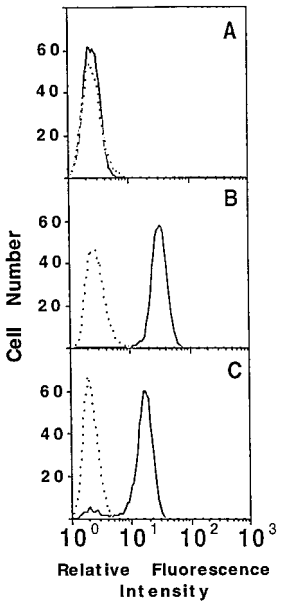

FIGURE 5: Expression of α3*β*1 on K562 cells. Parent K562 cells (A), wt α3-K562 cells (B), or mutant α3(153−165/α4)-K562 cells (C) were stained with control mouse IgG (···) or P1B5 (anti-α3) (—), followed by FITC-labeled goat anti-mouse IgG. Stained cells were analyzed by flow cytometry. The data suggest that K562 cells homogeneously express wt or mutant α3*β*1.

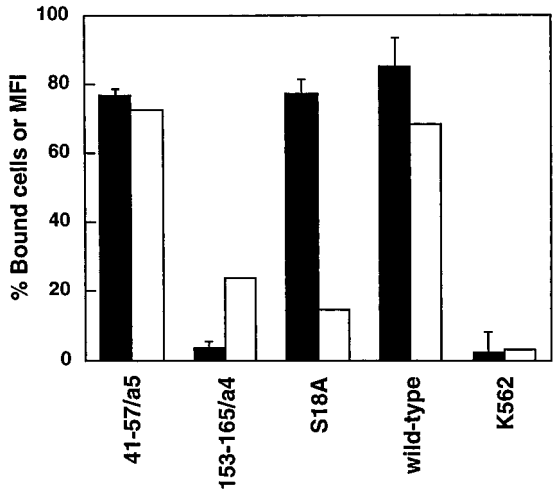

FIGURE 6: Adhesion to laminin-5 of cells expressing the *β*1/α3 domain-swapping mutants. Adhesion to laminin-5 of cloned K562 cells expressing wt or domain-swapping mutant α3 (solid bar); mean fluorescent intensity of cloned cells expressing wt or mutant α3 with mAb P1B5 (open bar). Adhesion was determined in the presence of 8A2, an activating anti-*β*1 mAb, and bound cells were quantified as described in the legend to Figure 1. Data are expressed as means ± SD of triplicate experiments. Cells were stained first with the anti-α3 mAb P1B5 and then with FITC-labeled goat anti-mouse IgG. The reactivity of other anti-α3 mAbs (e.g., 7A8) to the S18A mutant paralleled that of P1B5 (data not shown), indicating that the P1B5 epitope is not affected by the S18A mutation.

ECM (Figure 7). The T162A, T162F, and G163A mutations did not affect expression of either α3*β*1 or α3*β*1 binding to all of the mAbs tested (listed in Table 1). When these α3 mutants were transiently expressed on K562 cells, usually more than 50% of the transfected cells expressed α3 mutants (comparable to the level when wt α3 is transiently expressed), suggesting that these mutations do not induce drastic structural changes in α3*β*1. These results suggest that several amino acid residues that are clustered within or adjacent to the CNSNTDYLETGMC$^{153-165}$ sequence are critical for α3*β*1/laminin-5 interaction, supporting the data on the α3(153−165/α4) mutant.

Krukonis et al. (*17*) reported that mutation of Tyr-186 and Trp-188 in α3 to Ala blocks adhesion to laminin-5. These residues are located within another predicted loop in repeat 3 (residues 181−190) that has been reported to be critical for ligand binding in α4, α5, and αIIb (*14*, *41*, *42*). We studied the effect of point mutations (Y186A, N187A, N187Y, and W188A) on adhesion of α3-K562 cells to laminin-5. In the present study, these mutations did not block α3*β*1/laminin-5 interaction under the assay conditions used (Figure 7). We also introduced point mutations into predicted

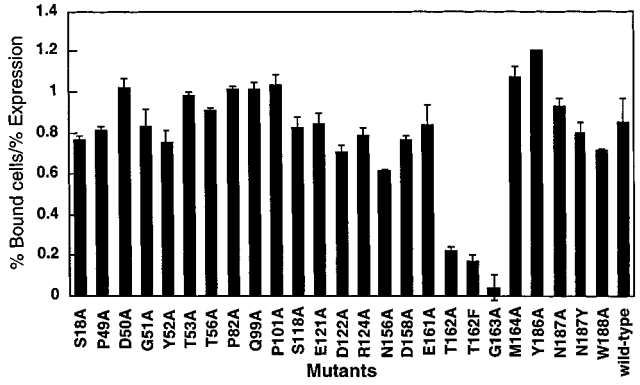

FIGURE 7: Alanine scanning mutagenesis of the α3 subunit. Adhesion to laminin-5 of K562 cells expressing mutant α3 was determined in the presence of 8A2, an activating anti-β1 mAb. Bound cells were quantified as described in the legend to Figure 1. Data are expressed as means of (% of bound cells per % of α3 positive cells) ± SD of triplicate experiments. Most of the stably transfected K562 cells were cloned after G418 selection to obtain high expressors. The T162A, T162F, G163A, M164A, and Y186A α3 mutants were transiently expressed on K562 cells. In transient expression studies, usually more than 50% of transfected cells express α3 mutants. Expression of α3 was determined by flow cytometry with mAb P1B5. The T157A and G190A mutants were not expressed on the cell surface.

loop structures in repeat 1 (P16A, S18A, P49A, D50A, G51A, Y52A, T53A, T56A, and P82A) and repeat 2 (Q99A, P101A, S118A, E121A, D122A, and R124A). These mutations did not block α3β1-mediated cell adhesion of laminin-5 ECM (Figure 7).

Effects of Mutations on Interaction between α3β1 and Invasin. We studied whether the α3 point mutations that are critical for binding to laminin-5 affect binding to invasin, another α3β1 ligand. We measured adhesion of K562 cells in the presence of mAb KH/72, an anti-α5 function-blocking antibody. As shown in Figure 8a, KH/72 completely blocks endogenous α5β1-mediated adhesion to invasin. Blocking of α3-K562 cell adhesion to invasin required both KH/72 and P1B5. Under the conditions used, α3β1-mediated adhesion to invasin with or without 8A2 is almost comparable (data not shown). The E161A (a positive control) and T162F mutants showed adhesion at levels comparable to that of wild type with or without 8A2. The T162A and G163A mutants showed lower adhesion without 8A2, but adding 8A2 restored their adhesion to invasin to the wild-type level. These results suggest that these mutations do not affect α3β1 adhesion to invasin when α3β1 is activated. This indicates (1) that residues critical for binding to laminin-5 and invasin may be different and (2) that mutation of Thr-162 and Gly-163 blocks adhesion to laminin-5 without altering structural integrity and other functions of α3β1.

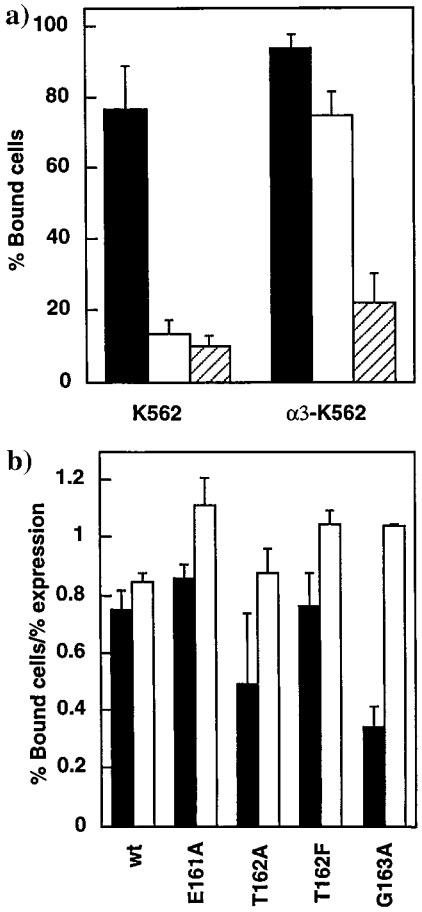

FIGURE 8: Effects of point mutations that affect adhesion to laminin-5 on adhesion to invasin. (a) Adhesion to invasin of K562 cells expressing α3 was determined in the absence of antibody (solid bar), in the presence of KH/72 (anti-α5, function-blocking) (open bar), or in the presence of both KH/72 and P1B5 (anti-α3, function-blocking) (hatched bar) at a coating concentration of 0.1 μg/mL invasin. Data are expressed as means ± SD of triplicate experiments. The results suggest that α5β1-mediated adhesion of parent K562 cells to invasin is completely blocked by KH/72. Blocking adhesion of α3-K562 cells to invasin requires both KH/72 and P1B5. At a higher coating concentration of invasin (more than 0.5 μg/mL), clear inhibition of α5β1-mediated adhesion by KH/72 was not observed. (b) Adhesion to invasin of K562 cells expressing mutant α3 was determined in the presence of KH/72 (solid bar) and both 8A2 and KH/72 (open bar). K562 cells stably expressing wt or mutant α3 were used for this adhesion assay. The data are expressed as the ratio % bound cells/% α3 positive cells (with anti-α3 mAb P1B5) ± SD of triplicate experiments. The % P1B5 positive cells are 99.3% (wt α3), 53.6% (E161A), 47.4% (T162A), 63.3% (T162F), 78.0% (G163A), and 1.9% (background in parent K562 cells). These results suggest that these mutations do not affect α3β1 adhesion to invasin when α3β1 is activated.

DISCUSSION

In this study we identified the α3 epitope at the boundary between repeats 1 and 2 (NITVKN$^{75-80}$) that is common to several function-blocking antibodies. The binding of antibodies to this epitope appears to be required to block α3β1 binding to laminin-5. This suggests that the ligand binding site is located sterically close to this epitope. We then found that swapping the CNSNTDYLETGMC$^{153-165}$ sequence at the boundary between repeats 2 and 3, or mutating Thr-162 and Gly-163 within this sequence blocks adhesion to laminin-5 ECM. These point mutations do not affect the binding to nearby epitopes of the anti-α3 mAb tested, suggesting that these mutations do not disturb the gross structure of α3. Interestingly, the T162A, T162F, and G163A mutations did not block binding of activated α3β1 to invasin, suggesting that invasin and laminin-5 may use different recognition mechanisms. Also, this finding suggests that these mutations do not induce drastic effects on the conformation and integrity of α3β1. It is possible that the G163A mutation works by changing the conformation of the predicted loop CNSNTDYLETGMC$^{153-165}$. It is possible that binding of antibodies to the epitope affects ligand binding by changing conformation of the ligand binding site.

Springer has proposed that the seven N-terminal repeats in integrin α subunits are folded into a β-propeller domain (*18*) containing seven four-stranded β-sheets arranged in a torus around a pseudosymmetrical axis. Integrin ligands are predicted to bind to the upper face of this β-propeller. Consistent with this model, the critical regions/residues for ligand binding in several non-I-domain integrin α subunits so far reported are all in the upper face of the proposed β-propeller domain. These include (1) the predicted loop sequences in α4, α5, and αIIb (*14, 41, 42*) that correspond to the GAPGAYNWKG$^{181-190}$ sequence of α3 (the 2-3 loop, Figure 4); (2) the three function-blocking αIIb mutations in Glanzmann's thrombasthenia [the P145A (*43*) and L183P (*44*) mutations, and insertion of two residues within the predicted loop spanning residues 147−166 of αIIb (*45*)]; and (3) the Thr-162 and Gly-163 residues of α3 (this study, the 4-1 loop, Figure 4). Also, the epitopes for function-blocking anti-α3 mAbs (NITVKN$^{75-80}$ sequence, this study) are in the upper face of the β-propeller. Interestingly, the NITVKN$^{75-80}$ sequence and the Thr-162/Gly-163 residues are close to each other in the proposed β-propeller model, although they are separated in the primary structure. This supports the β-propeller model, and emphasizes the importance of the Thr-162 and Gly-163 residues in ligand binding and its regulation. Recently Mould et al. reported that a double mutation of Ser-156/Trp-157 in the predicted loop of α5 corresponding to the α3 CNSNTDYLETGMC$^{153-165}$ sequence blocked binding of a ligand peptide RRETAWA (*46*) (but not the binding of fibronectin) to α5β1 (*47*). The Ser-156/Trp-157 residues are also in the upper face of the proposed β-propeller model. Thus, the predicted loops in the α subunit that are critical for ligand binding and its regulation may be clustered in the 2-3 loop and/or the 4-1 loop of repeat 3, which are in the upper face of the proposed β-propeller model (Figure 4), regardless of the ligand specificity of integrins. It is possible that these predicted loops are close to or within the ligand binding pocket of integrins. We recently found that an anti-αIIbβ3 ligand-mimetic mAb, which has an RGD-like sequence in its antigen binding site, recognizes both repeat 3 of αIIb and part of β3 (Puzon-McLaughlin, W., Kamata, T., and Takada, Y., manuscript submitted for publication). This supports the idea that repeat 3 of the α subunits may be close to or within the ligand binding pocket and make direct contact with ligands. Further structural and biochemical studies will be required to clarify this point.

Weitzman et al. (*48*) reported that α3β1 on K562 cells adheres to laminin-5 without activation (by activating anti-β1 mAbs such as 8A2 and TS2/16), and that activation did not further increase this adhesion. Another laboratory reported that α3β1 on K562 cells adheres to laminin-5 without activation, but that adhesion is increased by activation (*49, 50*). In our preliminary studies, the α3-K562 cells used in the present study were similar to those used by Weitzman et al. in their ability to adhere to laminin-5 under our assay conditions: both showed low affinity to laminin-5 without 8A2, and adhesion increased severalfold with activation (data not shown). Krukonis et al. reported that the Y186A and W188A α3 mutants did not adhere to laminin-5, and that the D122A α3 mutant showed enhanced adhesion to laminin-5 (*17*). In the present study, we did not detect any significant effects of these mutations on adhesion to laminin-5 under the assay conditions used (in the presence of 8A2). Krukonis et al. did not use an anti-β1 mAb to activate α3β1 on K562 cells, and therefore it is possible that they detected the effects of mutations at relatively low affinity conditions.

The recombinant cation-binding domain from the α5 subunit (residues 229−448, including repeats 4−7) has been reported to display a well-defined fold with a content of 30−35% α helix and 20−25% β strand, based on circular dichroism (*51*). This protein has also been reported to show cation-dependent interaction with RGD-containing ligands (*51*). These results are not consistent with the β-propeller model that has no α helix. Also, these results are not consistent with the previous and present mutagenesis and epitope mapping results in α3, α4, α5, or αIIb, since epitopes for function-blocking mAbs or critical residues for ligand binding have been localized within repeats 1−3, as described above. Further biochemical studies will be required to solve the inconsistency. Regardless of whether the proposed β-propeller model is correct, Thr-162 and Gly-163 of α3 are targets for further study of laminin-5/α3β1 interactions. The present ligand binding function-negative α3 mutants may be useful for studying ligand binding and signal transduction mediated by α3β1.

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