Regulation of Conformation and Ligand Binding Function of Integrin α5β1 by the β1 Cytoplasmic Domain*

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We have studied the role of the cytoplasmic domain in the conformation and affinity modulation of the integrin β1. Expression of a conformation-dependent anti-β1 antibody 15/7 correlates with activation in wild-type β1. Truncation of 16 carboxyl-terminal residues in the cytoplasmic domain (the 762t β1 mutant) induces constitutive expression of the 15/7 epitope at a high level (which probably reflects a major conformational change of the extracellular domain) but does not activate ligand binding. The dissociation of epitope expression and affinity suggests that the epitope expression reflects the conformation of nonligand binding sites of the extracellular domain of β1 but does not necessarily reflect that of the ligand binding sites. Indeed we discovered that the 15/7 epitope is in fact located in the nonligand binding region of β1 (within residues 354–425). The 762t mutant has apparently normal α/β association, suggesting that the overexpression of the 15/7 epitope is not due to α/β dissociation. The data suggest that the carboxy-terminal 16 residues of the β1 cytoplasmic domain are critical for properly modulating conformation and affinity of β1 integrins.

β1 integrins are members of the integrin supergene family of cell adhesion receptors that mediate cell-cell and cell-extracellular matrix interactions through interactions with multiple ligands (1–3). The ligand-binding affinity of β1 integrins is regulated by a variety of stimuli (4, 5), as is that of the β2 and β3 integrins (6–9). Qualitative changes in β1 integrin receptor functions play a critical role, for example, in leukocyte binding to endothelium (see Ref. 10 for review) and in inducing platelet aggregation (see Ref. 11 for review). The β1 subunit can regulate different cellular functions, because anti-β1 monoclonal antibodies (mAbs) can induce either comitogenic or antiproliferative signals to T-lymphocytes (12, 13). The ligand binding affinity of β1 integrins can also be nonphysiologically altered through inside-out signaling (e.g., by phorbol 12-myristate 13-acetate) or from outside of cells (e.g., by Mn2+ or activating anti-β1 mAb) (4, 5, 14–17). Binding activating mAbs to the regulatory epitope (18) induces a high affinity state in the receptor.

Probes that detect the physical changes in integrin structure that occur after cell activation (e.g., anti-β1 mAbs 15/7 (19)) are available for β1 integrins. The epitope for mAb 15/7 is induced by phorbol 12-myristate 13-acetate, Mn2+ (20), activating anti-β1 mAbs, and ligands or ligand-derived peptides (19). 15/7 recognizes only a subpopulation (5–10% of total) of the β1 integrin molecules, even when activated. The expression of the epitopes closely correlates with the ligand-binding ability of β1 integrins and thus serves as an indicator of conformational changes in functional integrin activation (19). The induction of the 15/7 epitope is reversed by inhibiting anti-β1 mAbs (19). However, mechanisms of activation and the accompanying conformational changes of β1 integrins are not fully understood.

The cytoplasmic domain of the β1 subunit has been shown to play a critical role in the association of integrin with the focal adhesion structure (20–22) and in signal transduction (Refs. 23–27; see Refs. 28 and 29 for review). The role of the β1 cytoplasmic domain in ligand binding functions and in conformational changes of β1 integrins has not been fully tested. In the present study we examined the potential role of the β1 cytoplasmic domain in conformation and ligand binding functions with a conformation-dependent anti-β1 mAb 15/7. Truncation of the 16 carboxyl-terminal residues of the cytoplasmic domain (the 762t β1 mutant) induced constitutive expression of the 15/7 epitope at a high level (which probably reflects a major conformational change of the extracellular domain) but did not activate ligand binding. The dissociation of epitope expression and affinity suggests that conformation of nonligand binding sites of the extracellular domain of β1 is reflected in the epitope expression, but this is not true for the ligand binding sites. Indeed the 15/7 epitope was located in the nonligand binding region of β1 (within residues 354–425). The data suggest that the carboxy-terminal 16 residues of the β1 cytoplasmic domain are critical for properly modulating conformation and affinity of β1 integrins.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—Anti-human β1 mAbs were kindly provided as follows: 4B4 (30), C. Morimoto (Dana-Farber Cancer Institute, Boston, MA); 8A2 (15), N. Kovach and J. Harlan (University of Washington, Seattle, WA); A1A5 and TS2/16 (31), M. E. Hemler (Dana-Farber Cancer Institute, Boston, MA); mAb 15/7 was prepared as described (19).

Construction of Plasmids—A cytoplasmic domainless 733t β1 mutant cDNA was constructed by inserting an XbaI linker into HindIII site of β1 cDNA clone B3 (2.9 kilobases) (32) after filling in reaction with Klenow fragment. The carboxy-terminal sequence of 733t β1 mutant is Gly-Leu-Ala-Leu-Leu-Leu-Ile-Trp-Lys-Leu (Stop) (Leu734–Lys778 is deleted). 739t, 753t, and 762t cytoplasmic domain truncation mutant β1 cDNAs were prepared by introducing stop codons at positions 740, 754, and 758 of β1, respectively, by site-directed mutagenesis (33). The presence of mutations was confirmed by DNA sequencing.

Stable Expression of Wild-type (wt) and Mutant β1 on CHO Cells—wt or mutant human β1 cDNA were subcloned into XbaI site of pB8-1 vector with an SV40 promoter (34, 35). CHO-K1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in a 6% CO2 incubator. 10 μg of the wt or
mutant \( \beta_1 \) pβ1-1 cDNA was transfected into CHO cells (8 \times 10^6 cells) with 1 μg of pFneo DNA containing a neomycin-resistant gene by electroporation. Transfected cells were maintained for 2 days in the above medium and then transferred to the medium supplemented with 700 μg/ml G418 (Life Technologies, Inc.). After 10–14 days, the resulting colonies were harvested, and those cells expressing human \( \beta_1 \) were cloned by cell sorting with mAb A1A5 in FACStar (Becton Dickinson).

Western Blotting Analysis of Chimeric \( \alpha_1 \)-\( \beta_1 \)-CHO cells expressing chimeric \( \beta_1 \) (110°C) were lysed in 1 ml of 20 mM Tris, 0.15 M NaCl, 1% Triton X-100, 0.05% Tween 20, pH 7.4. wt and chimeric \( \beta_1 \) integrin were first immunopurified with anti-human \( \beta_1 \) mAb A1A5 immobilized to Sepharose. After washing the A1A5-Sepharose, the bound materials were recovered by boiling in SDS-polyacrylamide gel electrophoresis buffer containing 1% SDS (w/v) for 5 min and separated by SDS-polyacrylamide gel electrophoresis in 7% gels under nonreducing conditions. Proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), and the membrane was blocked by incubating with 1% dry milk proteins for 1 h at room temperature. The membrane was used for Western blotting analysis with anti-\( \beta_1 \) mAbs 15/7 or TS2/16. Goat anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad) and an ECL kit (Amersham Corp.) were used to detect antibody binding.

Mapping the Epitope for mAb 15/7 Using Interspecies Chimeras—cDNAs for human/mouse interspecies chimeras of \( \beta_1 \) were prepared and used to stably transfect CHO cells as described previously (18). CHO cells expressing \( \beta_1 \) chimeras were then cloned to obtain cells expressing high level \( \beta_1 \) chimeras by cell sorting in FACStar. For flow cytometry, CHO cells were first incubated with 8A2 (activating anti-\( \beta_1 \) mAb) or 4B4 (inhibiting anti-\( \beta_1 \) mAb) and then incubated with FITC-labeled mAb 15/7 or mouse IgG. Stained cells were analyzed using FACScan (Becton Dickinson).

Binding of FITC-labeled Fn to Cells Expressing Human \( \beta_1 \) Mutants—Fn was labeled with FITC essentially as described (36). Briefly, Fn (0.5 mg) and 1.5 mg/ml of FITC isomer 1 on cell line (Sigma) in 1 ml of 0.1 M NaHCO₃, pH 9.3, were incubated for 1 h at room temperature in the dark. Free FITC was removed using a Sephadex-G25 PD-10 column (Pharmacia Biotech Inc.) equilibrated with 10 mM sodium phosphate, 0.14 M NaCl (PBS). The concentration of FITC-labeled Fn was calculated using [Fn] = \( [\text{A}_{280} - (0.35 \times \text{A}_{232})]/2.23 \), where 1.23 is the extinction coefficient for purified Fn. The molar fluorescent/protein ratio was calculated from A₂₈₀ with the extinction coefficient of 200 (37). The fluorescent/protein value for FITC-Fn used in this experiment was 4.8.

Cells were harvested with 3.5 ml EDTA in PBS and washed with PBS. Cells were first incubated with mAb 4B4 or 8A2 in DMEM at saturating concentrations (1000 times dilution of ascites) for 30 min on ice. After washing once with PBS, cells were incubated with FITC-Fn (at a final concentration of 25 μg/ml) in DMEM for 30 min on ice. After washing once with PBS, cells were suspended in PBS and analyzed using FACScan.

Other Methods—Flow cytometric analysis and immunoprecipitation of surface \( ^{125}\text{I} \)-labeled cell extracts with specific mAbs were performed as described (32).

RESULTS

Effects of Truncation of the \( \beta_1 \) Cytoplasmic Domain on the 15/7 Epitope Expression—We studied the potential roles of the \( \beta_1 \) cytoplasmic domain in the conformational changes of the extracellular domain using mAb 15/7 as a probe. CHO cells expressing \( \beta_1 \) mutants with varying lengths of cytoplasmic domain (733t, 739t, 753t, and 762t) were cloned to obtain high expressers. FITC-labeled anti-mouse IgG was used to detect binding of mAb 15/7 (Fig. 1). Interestingly, these truncation mutants overexpress the 15/7 epitope at levels close to nonconformation-dependent mAb A1A5 epitope (Fig. 2). The ratio of mean fluorescent intensity with mAb 15/7 to that with mAb A1A5 (normalized 15/7 expression) was 0.65 for 762t, 0.84 for 753t, 0.89 for 739t, and 1.26 for 733t. This is in contrast to a normalized expression of 0.11 for wt \( \beta_1 \) (Fig. 2).

We measured the reactivity of the truncation mutants with mAb 15/7 in the presence of activating or inhibiting anti-\( \beta_1 \) mAbs using flow cytometry. For this purpose we used mAb 15/7 directly labeled with FITC. The expression of the 15/7 epitope on these truncation mutants was not further increased by an activating mAb nor was it decreased by an inhibiting mAb (Fig. 3). These data suggest that truncation of the cytoplasmic domain of \( \beta_1 \) induces a conformation in the extracellular domain that overexposes the 15/7 epitope but is not responsive to activating or inhibiting antibodies.

Ligand Binding Function of Mutants with Truncation of the \( \beta_1 \) Cytoplasmic Domain—To determine if the levels of the 15/7 epitope in the truncation mutants reflect those of ligand binding function, we examined the binding of FITC-labeled Fn to cells expressing mutants. Ligand binding specific to human \( \beta_1 \) subunit was measured using activating (8A2) or inhibiting (4B4) anti-human \( \beta_1 \) antibodies. The difference between binding in the presence of 8A2 and binding in the presence of 4B4 reflects binding to human \( \beta_1 \) subunit but not to hamster \( \beta_1 \) subunit. Significant binding of FITC-labeled Fn specific to human \( \beta_1 \) subunit was observed with cells expressing wt human \( \beta_1 \) (Fig. 4A) but not with parent CHO cells. FACScan binding to wt \( \beta_1 \) subunit was increased by 8A2, and the increase was completely blocked by either EDTA (1 mM) or Arg-Gly-Asp-Ser (RGDS) peptide (1 mg/ml) in the mixture (data not shown). In contrast, the truncation \( \beta_1 \) mutants only slightly increased ligand binding function in response to 8A2 (Fig. 4A). The levels of Fn binding were
compared with those of human β1 expression (Fig. 4B). The truncation mutants clearly show much lower response to activation than wt β1. Fn binding to wt β1-CHO cells without any antibody is almost identical to that with 4B4 in each case (data not shown), suggesting that both endogenous hamster and human β1/α5 are in a default low affinity state in the assay conditions. Although the binding of FITC-Fn to the 733t mutant appears to be much higher than its binding to the other mutants, that is not the case, because the level of β1 expression on the 733t β1-CHO cells is much higher than that of the other mutants (Fig. 4B) and because relative fluorescence intensity is shown in the log scale in Fig. 4A. The data indicate that the truncation mutant reduces the ability to modulate ligand binding in response to stimulation. The truncation mutants remain at a low affinity level on activation despite high level expression of 15/7.

We analyzed the mutants by immunoprecipitation with anti-human β1 mAb A1A5 to examine the effects of truncation on α-β association. The 762t mutant β1 showed association with hamster α subunit (mainly α5) at a level similar to that of wt β1. The other β1 mutants (753t, 739t, and 733t) have reduced association with endogenous hamster α under the detergent conditions used (1% Triton X-100, 0.05% Tween 20) (Fig. 5). The level of association with the α subunits roughly correlates with the lengths of the remaining cytoplasmic domains, suggesting that the β1 cytoplasmic domain may also be required for α-β association. These data indicate that overexpression of the 15/7 epitope or the reduced Fn binding may not be simply due to α-β dissociation, because the 762t mutant shows α-β association at a normal level but already shows overexpression of the 15/7 epitope and reduced ligand affinity.

Localization of the 15/7 Epitope on the β1 Subunit—We mapped the conformation-dependent 15/7 epitope using CHO cells expressing wt and human/mouse chimeric β1 to identify the region of β1 that is involved in conformational changes on activation. The rationale for the mapping strategy is that although there is more than 85% homology between human and mouse β1, mAb 15/7 recognizes human β1 but not mouse β1. This strategy has been used to localize epitopes for other anti-integrin antibodies (18, 38–41). Previously published CHO cell lines expressing β1 chimeras h587/m (residues 1–587 from human β1 and 588–778 from mouse β1), h425/m, and h354/m (18) (Fig. 6) were cell-sorted to obtain high expressers, because 15/7 reacts with only 5–10% of the surface β1 integrins, even when activated. The levels of expression of the β1 chimeras are comparable with those of endogenous hamster β1 subunit (data not shown).

The reactivity of mAb 15/7 to chimeric β1 was first examined using flow cytometry. CHO cells expressing β1 chimeras were
incubated with FITC-labeled 15/7 mAb in the presence of 8A2 (activating mAb) or 4B4 (inhibiting mAb). As shown in Fig. 7, 8A2 induced 15/7 epitopes in wt, h587/m, and h425/m but not in h354/m. Endogenous hamster \( \beta_1 \) on CHO cells showed weak reactivity to mAb 15/7. Other \( \beta_1 \) chimeras, h304/c (residues 1–304 from human \( \beta_1 \) and 305–778 from chicken \( \beta_1 \)) and h189/c (uncloned, more than 50% positive in human \( \beta_1 \) expression) showed fluorescence-activated cell sorter profiles similar to those of h354/m \( \beta_1 \) (data not shown). The reactivity of mAb 15/7 was then tested by Western blotting of immuno-purified chimeric \( \beta_1 \) with mAbs 15/7 and TS2/16 (as positive control). Fig. 8 shows that mAb 15/7 recognizes wt, h587/m, and h425/m \( \beta_1 \) but not h354/m \( \beta_1 \), whereas TS2/16 recognizes all these \( \beta_1 \) molecules. These data show that the residues 354–425 of \( \beta_1 \) are critical for the binding of mAb 15/7. These residues are located close to the boundary between the global

**DISCUSSION**

This paper establishes that truncation of more than 16 carboxyl-terminal residues of the \( \beta_1 \) cytoplasmic domain results in drastic effects on the conformation and affinity of the \( \beta_1 \) integrin's extracellular domain (these effects include overexpression of the conformation-dependent 15/7 epitope and greatly reduced \( \alpha_5 \beta_1 \) ligand binding affinity). These findings emphasize the critical role of the cytoplasmic domain of integrin \( \beta_1 \) in the conformation and affinity of the extracellular domain. The conformational change of the extracellular domain due to truncation of the cytoplasmic domain has not been reported, probably because it is not detected by nonconformation-dependent
anti-β1 mAbs. The secondary structure analysis, performed using the GCG PEPTIDESCRIPTR program, predicts that the carboxyl-terminal portion of the integrin β1 cytoplasmic domain (residues 763–778) contains a β-sheet structure (not shown). Therefore, the predicted β-sheet structure may be critical for affinity modulation and conformational changes in response to activation or inhibition. Although truncation of more than 25 residues of the cytoplasmic domain results in reduced α-β association in the present study, it is not likely that dissociation of the α and β subunits is directly related to an overexpression of the 15/7 epitope and reduced ligand binding affinity. This is because the 762t mutant β1 (with truncation of 16 residues) associates with α at a level similar to that of wild-type β1 but shows an overexposure of the 15/7 epitope and reduced ligand binding affinity.

The 15/7 epitope expression correlates with the ligand binding affinity of wt β1 integrins (19), and only a slight increase/decrease in the 15/7 signal is observed on activation/inhibition of wt β1 integrins. The 762t (and other truncation) β1 mutants constitutively express the epitope at a high level, whereas the mutant has constitutively low affinity. The dissociation of the epitope expression and ligand binding affinity suggests that the 15/7 epitope expression reflects conformation of nonligand binding sites of the β1 extracellular domain but does not necessarily reflect conformation of the ligand binding sites. Indeed, the 15/7 epitope is located in the middle of the β1 subunit (nonligand binding region) as shown in this study. This is in contrast to ligand-mimetic antibodies (e.g., PAC-1 for αIβ3) that reflect function and affinity of the ligand-binding sites. The ligand-mimetic antibodies have RGD-like RYY sequences in antigen binding sites and are believed to bind to the ligand binding sites (43–45).

Williams et al. (46) recently proposed that the predicted amino-terminal α-helical structure of the cytoplasmic domain may be involved in inside-out or outside-in signal transduction. The amino-terminal portion is more likely to be involved in α-β association rather than regulation of integrin affinity in the present system, because 1) truncation of more than 25 residues of the β1 cytoplasmic domain results in a reduced α-β association and 2) consistently, truncation of the carboxyl-terminal portion of the β1 cytoplasmic domain was enough to induce a low affinity state of α5β1 in the present study. The interaction between α and β cytoplasmic domains of αIIbβ3 has been reported using synthetic peptides (47, 48). Truncation of the cytoplasmic domain of other β subunits (e.g., αIIbβ3) has not been reported to result in a decrease in α-β association. One possibility is that association in the extracellular domain is strong enough to support stable association of the heterodimer. If this is the case, the effect of cytoplasmic domain truncation may not be detected by immunoprecipitation. Indeed, soluble αIIbβ3 without transmembrane and cytoplasmic domains still makes stable heterodimer (49–51) and truncated β3 associates with αIIb on immunoprecipitation (52).

In the present study, truncation of more than 16 residues of the β1 cytoplasmic domain resulted in a constitutive low affinity state. Although there is a consensus that partial truncation of the cytoplasmic domain of β results in a constitutive low affinity state, the effects of complete truncation appear to be dependent on integrin species. Truncation of the cytoplasmic domain of the β2 subunit of the leukocyte integrin αLβ2 eliminated binding to intercellular adhesion molecule-1 and sensitivity to phospholipids esters (53, 54). Partial truncations of the β3 cytoplasmic domain blocked inside-out signaling, but complete truncation caused constitutive activation in CHO cells, as assessed by the binding of a ligand mimetic antibody, PAC-1 (55). A partial truncation mutant of the β7 cytoplasmic domain of 49/7 displayed no ligand binding activity to fibronectin and vascular cell adhesion molecule-1, whereas the complete truncation mutant of β7 was constitutively active for all ligands and displayed greater affinity than the wt β7 in the B cell lymphoma 38C13 (56). It remains to be seen whether the discrepancy in the effects of complete truncation on ligand binding may be due to different assays used for ligand/integrin interaction (e.g., adhesion to immobilized ligand, binding to soluble ligands, or binding of ligand-mimetic antibodies) or due to different cellular background (e.g., adherent cells or nonadherent cells). Another possibility is that ligand binding sites or binding mechanisms may be different for each ligand. We cannot rule out this possibility because very limited information is available on this subject.

Epitope mapping using interspecies chimeric molecules has been used to localize epitopes for function-blocking antibodies in other integrin subunits (18, 38–41). Point mutations within the epitope regions actually block binding of antibodies and/or ligands (18, 38, 39, 57), proving the rationale of the strategy to be correct. The present epitope mapping study establishes that mAb 15/7 defines a novel cryptic conformation-dependent epitope, which is localized in a nonligand binding region within residues 354–425, between one region containing both the putative ligand binding domains (58–60) and the regulatory epitope (18) and a second region containing the cysteine-rich repeats (residues 446–615) (61) (Fig. 6). The 15/7 epitope may be located close to the boundary between the amino-terminal global domain and the carboxyl-terminal stalk region of β1 based on the electron microscopic studies of α5β1 (42).

Recently Bazzoni et al. localized the epitope for another conformation-dependent anti-β1 mAb 9E67 within residues 495–602 of β1 using interspecies chimeras (62). They also claimed that mAb 15/7 recognizes a regulatory epitope on β1 (residues 207–218) (18), because 13, an inhibitory mAb that recognizes the regulatory epitope of β1, competes with mAb 15/7 for binding to β1. It is likely that mAb 13 induced an inactive conformation of β1 and thereby indirectly turned off

![Fig. 8. Western blotting analysis of chimeric β1 with mAb 15/7.](image-url)
expression of the 15/7 epitope. Indeed, mAbs 8A2 and TS2/16, activating mAbs that recognize the regulatory epitope, induce the 15/7 epitope, whereas mAb AIIB2, an inhibitory mAb that also recognizes the 15/7 epitope, represses the 15/7 epitope (19). Clearly, 8A2 does not compete with 15/7 for binding to β1.

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