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Association of the Membrane Proximal Regions of the α and β Subunit Cytoplasmic Domains Constrains an Integrin in the Inactive State*

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The adhesiveness of integrins is regulated through a process termed "inside-out" signaling. To understand the molecular mechanism of integrin inside-out signaling, we generated K562 stable cell lines that expressed LFA-1 ($\alpha_L\beta_2$) or Mac-1 ($\alpha_M\beta_2$) with mutations in the cytoplasmic domain. Complete truncation of the β_2 cytoplasmic domain, but not a truncation that retained the membrane proximal eight residues, resulted in constitutive activation of $\alpha_L\beta_2$ and $\alpha_M\beta_2$, demonstrating the importance of this membrane proximal region in the regulation of integrin adhesive function. Furthermore, replacement of the α_L and β_2 cytoplasmic domains with acidic and basic peptides that form an α-helical coiled coil caused inactivation of $\alpha_L\beta_2$. Association of these artificial cytoplasmic domains was directly demonstrated. By contrast, replacement of the α_L and β_2 cytoplasmic domains with two basic peptides that do not form an α-helical coiled coil activated $\alpha_L\beta_2$. Induction of ligand binding by the activating cytoplasmic domain mutations correlated with the induction of activation epitopes in the extracellular domain. Our data demonstrate that cytoplasmic, membrane proximal association between integrin α and β subunits, constrains an integrin in the inactive conformation.

Integrins are heterodimeric adhesion molecules that mediate important cell-cell and cell-extracellular matrix interactions. To date, 25 different integrin αβ heterodimers have been reported (1). The leukocyte integrin subfamily consists of four members that share the common β_2 subunit (CD18) but have distinct α subunits, α_L (CD11a), α_M (CD11b), α_X (CD11c), and α_D for LFA-1, Mac-1, p150,95 and α_D/β_2, respectively (2–4). LFA-1 is expressed on all leukocytes and is the receptor for three Ig superfamily members, intercellular adhesion molecule-1, -2, and -3 (ICAM[1]-1, -2 and -3). Mac-1 and p150,95 are primarily expressed on myeloid lineage cells and bind ligands including ICAM-1, the complement component iC3b, and fibrinogen. The leukocyte integrins mediate a wide range of adhesive interactions that are essential for normal immune and inflammatory responses (5). Patients with lymphocyte adhesion deficiency have defective expression of leukocyte integrins on the cell surface because of mutations in the common β_2 subunit. This disease causes an inability of phagocytic cells to bind to and migrate across the endothelium at sites of inflammation, resulting in severe bacterial and fungal infections (3).

The adhesiveness of leukocyte integrins is dynamically regulated in cells by cytoplasmic signals, a process termed inside-out signaling. For example, T-cell receptor cross-linking or activation of leukocytes with phorbol esters rapidly increases adhesiveness through LFA-1 (6–8). The enhanced adhesion is transient, and by 30 min after stimulation, cells lose their ability to bind to ICAM-1. This may provide a mechanism for regulating T cell adhesion and de-adhesion with antigen-presenting and target cells. In addition to activation by intracellular signals, divalent cations can directly modulate the ligand-binding function of leukocyte integrins (9–11). Activation can also be mimicked by certain antibodies that bind to the extracellular domain of the leukocyte integrins (12–15).

A key question on integrins is how signals are transduced from the cytoplasm to the ligand-binding site in the extracellular domain. The integrin α and β subunits are both type I transmembrane glycoproteins. Electron microscopy of integrins reveals an overall structure with a globular headpiece connected to the plasma membrane by two long stalks each about 16 nm long (16). The two stalks correspond to the C-terminal portions of the α and β subunits. The headpiece binds ligand and contains the more N-terminal domains, including a predicted β-propeller domain and I domain in the α subunit and an I-like domain in the β subunit. Both conformational change (affinity regulation) and receptor clustering in the membrane (avidity regulation) have been proposed as mechanisms for the enhancement of integrin adhesiveness through inside-out signaling (17–20). Conformational change in integrin I domains has been demonstrated in structural studies (21–23) and found to regulate ligand binding affinity (23–25).

The importance of integrin α and β subunit cytoplasmic domains in inside-out signaling has been demonstrated by mutagenesis studies. Whereas partial deletions of the α_L cytoplasmic domain have no effect on binding to ICAM-1, complete truncation of the cytoplasmic domain or internal deletion of the conserved membrane proximal GFFKR sequence constitutively activates LFA-1 (26, 27). Truncation of the α_{IIb} cytoplasmic domain before, but not after the conserved GFFKR sequence renders the $\alpha_{IIb}\beta_3$ integrin constitutively active (28, 29). These findings demonstrate the importance of the membrane proximal α subunit GFFKR sequence in the regulation of integrin adhesiveness. Partial truncations of the β_3 cytoplasmic domain maintain $\alpha_{IIb}\beta_3$ in a low affinity state, but complete truncation or deletion of the membrane proximal seven residues causes constitutive ligand binding by $\alpha_{IIb}\beta_3$, indicating that this mem-

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[1] The abbreviations used are: ICAM, intercellular adhesion molecule; mAb, monoclonal antibody; PCR, polymerase chain reaction; FACS, fluorescent-activated cell sorter.

brane proximal region of the β_3 subunit is required to maintain $\alpha_{IIb}\beta_3$ in a low affinity state (30). It has been suggested that interactions between the α and β subunit cytoplasmic/transmembrane domains that include complementary negatively and positively charged residues restrain integrins in an inactive state (29, 31). Several proteins that associate with integrin cytoplasmic domains have been identified (32–36); however, how these integrin-associated proteins function in physiological activation of integrins remains unclear.

Here we test the hypothesis that association between the membrane proximal regions of the integrin α and β cytoplasmic domains constrains an integrin in the inactive state. We demonstrate that the membrane proximal region of the β_2 cytoplasmic domain plays an important role in the formation of cell surface $\alpha\beta$ heterodimers and maintenance of $\alpha_L\beta_2$ and $\alpha_M\beta_2$ in an inactive state. Replacement of the α_L and β_2 cytoplasmic domains with acidic and basic peptides that form an α-helical coiled coil renders the integrin inactive in cell types in which wild type $\alpha_L\beta_2$ is either basally active or inactive, whereas replacement of the cytoplasmic domain with two basic peptides that do not form a heterodimer renders the integrin active in cell types in which wild type $\alpha_L\beta_2$ is either basally active or inactive. Our findings directly demonstrate that association between the membrane proximal segments of the α and β cytoplasmic domains regulates ligand binding by integrin extracellular domains.

MATERIALS AND METHODS

Monoclonal Antibodies—The murine mAbs TS1/22, TS2/4 to α_L (CD11a), CBR LFA-1/7 and CBR LFA-1/2 to β_2 (CD18), CBRM1/33, CBRM1/20 and CBRM1/5 to α_M (CD11b), and the nonbinding IgG X63 were described previously (15, 37–39). KIM127 (13) was kindly provided by M. Robinson (Celltech Limited, England). mAb m24 (9) was a kind gift from N. Hogg (Imperial Cancer Fund, England). mAb 2H11 (40) was generously provided by H-C Chang (Dana-Farber Institute, Boston).

Construction of Mutant α_L and β_2 Subunits—β_2 truncation mutants $\beta_2$710* and $\beta_2$702* were generated by introducing a stop codon at 710 and 702 in the β_2 subunit, respectively (the 22 amino acid signal sequence was not included in β_2 numbering; Fig. 1*A*). A *Not*I restriction site was designed in the downstream PCR primer after the stop codon. The PCR upstream primer corresponded to β_2 cDNA sequences from 1651–1672. The wild-type β_2 in plasmid Ap^{r}M8 (41) was used as template for PCR reaction. The PCR product was cut with *Bst*BI at nucleotide 1977 and *Not*I, and swapped with the corresponding *Bst*B1-*Not*I fragment from wild-type β_2 in Ap^{r}M8.

The $\alpha_L$acid and $\alpha_L$base constructs were generated by fusing a 30-amino acidic peptide or a 30-amino basic peptide (42) to the extracellular and transmembrane domains of the α_L subunit following residue Tyr-1087 (Fig. 1*A*). The constructs were made by overlap extension PCR (43, 44) using the nucleotide sequences of the acidic and basic peptides (40). A stop codon was designed at the end of the peptide, and following the stop codon was an *Sph*I site. The outer left PCR primer was 5′ to the *Bst*XI site at nucleotide 2963 of the α_L cDNA. Two rounds of overlap PCR were performed. The final PCR product was cut with *Bst*XI and *Sph*I, and the *Bst*XI-*Sph*I fragment was swapped into the same sites of wild-type α_L cDNA in plasmid Ap^{r}M8. The unique *Nhe*I site in the acidic and basic peptide nucleotide sequences was used for mutant identification.

The $\beta_2$base construct was made by fusing the basic peptide (42) to residue Trp-701 at the end of the putative β_2 transmembrane domain with overlap extension PCR (Fig. 1*A*). The PCR strategy was similar to that for making the $\alpha_L$acid and $\alpha_L$base constructs. The final PCR product was digested with *Bst*BI and *Not*I, and the *Bst*BI-*Not*I fragment was swapped into the same sites in wild-type β_2 cDNA contained in plasmid Ap^{r}M8. All mutations were verified by DNA sequencing.

Transient and Stable Transfection—Transient transfection of 293T cells was described previously (45). Stable transfection of K562 cells and maintenance of stable cell lines were as described previously (27, 45).

Immunofluoresence Flow Cytometry—Flow cytometry of cells was described previously (27). Briefly, cells (10^5) were incubated with primary antibody in 100 μl of L15/fetal bovine serum on ice for 30 min, except for KIM127 and m24. Incubation with mAbs KIM127 and m24 was carried out at 37 °C for 30 min (9, 13). mAbs, except for X63, were used as purified IgG at 10 μg/ml. The nonbinding IgG X63 was used at 1:20 dilution of hybridoma supernatant. Cells were then washed twice with L15/fetal bovine serum and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (heavy and light chain, Zymed Laboratories Inc. Laboratories, San Francisco, CA) for 30 min on ice. After washing, cells were resuspended in cold phosphate-buffered saline and analyzed on a FACScan (Becton Dickinson, San Jose, CA).

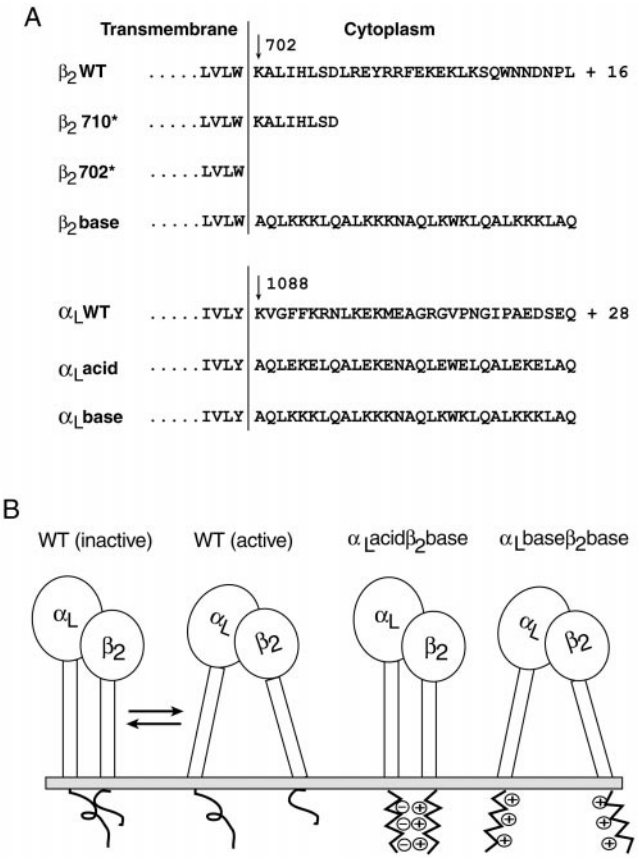

FIG. 1. **Schematic diagram of α_L and β_2 subunit mutants.** *A,* the sequences of the mutants are shown in the membrane proximal regions. Numbers to the right (+16 and +28) indicate the number of C-terminal residues not shown. *B,* scheme for regulation of integrin adhesiveness by cytoplasmic domain association/dissociation with the acid and base peptide fusions. The acid and base peptides are shown as (− − −) and (+ + +), respectively.

Metabolic Labeling and Immunoprecipitation—Metabolic labeling and immunoprecipitation was described previously (27). Briefly, 2×10^7 cells in 4 ml of labeling medium (methionine and cysteine-free RPMI 1640 containing 15% dialyzed fetal bovine serum) were labeled with 0.4 mCi of [^{35}S]methionine and cysteine (ICN Biochemicals) overnight in a 37 °C incubator. Labeled cells were lysed, and the lysate was incubated with antibody-coupled-Sepharose beads overnight at 4 °C. The immunoprecipitates were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and fluorography.

Cell Adhesion—ICAM-1 was purified from human tonsil, and coated on 96-well plates as described previously (27). Human complement component iC3b was purchased from CalBiochem and coated at 10 μg/ml. Cell adhesion to immobilized ligand was described previously (27, 45). Bound cells were expressed as a percentage of total input cells.

RESULTS

The Membrane Proximal Region of the β_2 Cytoplasmic Domain Regulates Integrin $\alpha\beta$ Heterodimer Formation on the Cell Surface—To examine the role of the membrane proximal region of the β_2 cytoplasmic domain in the regulation of $\alpha\beta$ heterodimer formation and ligand binding activity, we generated β_2 cytoplasmic domain truncation mutants $\beta_2$710* and $\beta_2$702*. $\beta_2$710* retained the membrane proximal sequence KALIHLSD, whereas $\beta_2$702* contained a complete truncation of the β_2 cytoplasmic domain (Fig. 1*A*). The β_2 truncation mutants were

TABLE I
Flow cytometric measurement of $\alpha_L\beta_2$ expression on the surface of 293T transfectants

Wild-type or truncated β_2 was transiently coexpressed with wild-type α_L in 293T cells. Included for comparison is the α_L cytoplasmic domain truncation mutant α_L1090* (27) coexpressed with wild-type β_2. Cell surface expression of heterodimeric $\alpha_L\beta_2$ was determined by flow cytometry using mAb TS1/22 to α_L, mAb TS2/4 to α_L in the $\alpha_L\beta_2$ complex, and mAb CBR LFA-1/7 to β_2. Mean fluorescence intensity shown was subtracted by that of the nonbinding IgG X63. Data are mean ± difference from the mean of two independent experiments.

Transfectants	Mean fluorescence intensity		
	TS1/22	TS2/4	CBR LFA-1/7
$\alpha_L\beta_2$	365 ± 19	387 ± 58	381 ± 43
$\alpha_L\beta_2$710*	339 ± 12	329 ± 37	314 ± 13
$\alpha_L\beta_2$702*	112 ± 9	114 ± 14	120 ± 11
α_L1090*β_2	132 ± 18	115 ± 5	110 ± 9

transiently coexpressed with wild-type α_L in 293T cells. Cell surface expression of heterodimeric $\alpha_L\beta_2$ was determined by indirect immunofluorescence staining with mAbs TS1/22 to α_L, CBR LFA-1/7 to β_2, and TS2/4 to α_L in the $\alpha_L\beta_2$ complex (Table I). The level of cell surface heterodimeric $\alpha_L\beta_2$710* was comparable with that of wild-type $\alpha_L\beta_2$. However, the level of $\alpha_L\beta_2$702* was greatly reduced (Table I). Complete truncation of the β_2 cytoplasmic domain also greatly reduced cell surface expression of the $\alpha_M\beta_2$702* heterodimer (data not shown). Thus, the membrane proximal sequence KALIHLSD plays a role in the formation of cell surface $\alpha\beta$ heterodimer. Complete truncation of the α_L cytoplasmic doman in mutant α_L1090* reduced surface expression as previously described (27). The reduction in expression of α_L1090* β_2 was similar to that seen with $\alpha_L\beta_2$702* (Table I).

Complete Truncation of the β_2 Cytoplasmic Domain Results in Constitutive Ligand Binding by $\alpha_L\beta_2$ and $\alpha_M\beta_2$—To examine the effect of β_2 cytoplasmic domain truncations on ligand binding by $\alpha_L\beta_2$, the β_2 truncation mutants $\beta_2$710* and $\beta_2$702* were stably coexpressed with wild-type α_L in K562 cells. Clones of wild-type $\alpha_L\beta_2$, $\alpha_L\beta_2$710*, and $\alpha_L\beta_2$702* transfectants that expressed similar levels of surface $\alpha_L\beta_2$, as determined by flow cytometry (Fig. 2*A*), were selected and tested for their ability to bind to purified ICAM-1. Transfectants that expressed wild-type $\alpha_L\beta_2$ and $\alpha_L\beta_2$710* showed low basal binding to ICAM-1; however, binding was greatly increased by the activating mAb CBR LFA-1/2 to the β_2 subunit (Fig. 3*A*). By contrast, $\alpha_L\beta_2$702* showed strong constitutive binding to ICAM-1, and the level of binding was comparable with that of the constitutively active α_L truncation mutation α_L1090*. CBR LFA-1/2 did not further enhance ligand binding by $\alpha_L\beta_2$702* and α_L1090*β2, suggesting that these mutants are fully active without activation. All binding was specific, as shown with inhibition by mAb TS1/22 to the α_L I domain and by comparison to mock transfectants.

The β_2 cytoplasmic domain truncation mutants $\beta_2$710* and $\beta_2$702* were also stably coexpressed with the wild-type α_M subunit in K562 cells. Clones of K562 transfectants that expressed similar levels of cell surface $\alpha_M\beta_2$ heterodimer, as determined by flow cytometry (Fig. 2*B*), were tested for binding to immobilized iC3b. K562 transfectants that expressed wild-type $\alpha_M\beta_2$ or $\alpha_M\beta_2$710* did not bind to iC3b in the absence of the activating mAb CBR LFA-1/2. By contrast, $\alpha_M\beta_2$702* bound strongly to iC3b without activation. Binding was specific, because it was inhibited by mAb CBRM1/33 to the α_M I domain.

Thus, complete truncation of the β_2 cytoplasmic domain constitutively activates ligand binding by $\alpha_L\beta_2$ and $\alpha_M\beta_2$, whereas partial truncation that retains the membrane proximal eight residues does not. These results suggest that the membrane proximal region in the β_2 cytoplasmic domain constrains β_2 integrins in the inactive state.

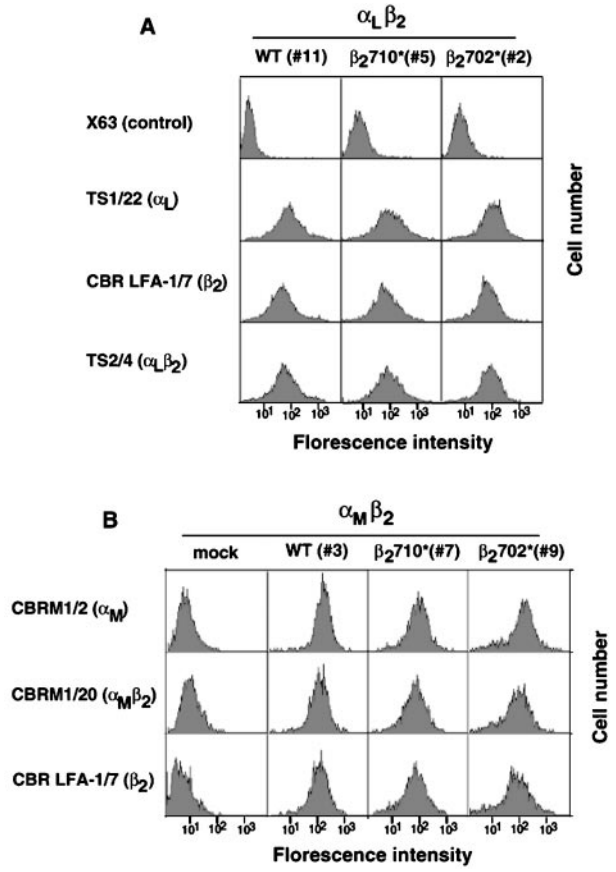

FIG. 2. **Expression of truncation mutants on the surface of K562 cell transfectants.** Wild-type α_L (*A*) or α_M (*B*) was coexpressed with wild-type (*WT*) β_2, truncation mutant $\beta_2$710* or $\beta_2$702* in K562 cells, or cells were transfected with vector alone (*mock*). Cell surface expression of the $\alpha_L\beta_2$ (*A*) and $\alpha_M\beta_2$ (*B*) complexes was determined by immunofluorescence flow cytometry. *Numbers in parentheses* after the transfectant names are clone numbers. mAbs and their specificity are indicated on the *left*. *X63* is a nonbinding myeloma IgG control.

Activating β_2 Truncation Mutations Expose Activation-dependent Epitopes in $\alpha_L\beta_2$ and $\alpha_M\beta_2$—mAb m24 has been used as a reporter for $\alpha_L\beta_2$ activation (10, 27, 46). Recently, mAb m24 has been mapped to the I-like domain of the β_2 subunit (47). mAb KIM127 recognizes an epitope in the β_2 stalk region that becomes exposed upon receptor activation (48). We therefore tested expression of the m24 and KIM127 epitopes by $\alpha_L\beta_2$ containing β_2 truncation mutations. There was little expression of the m24 epitope by wild-type $\alpha_L\beta_2$ or $\alpha_L\beta_2$710* (Table II). However, the truncation mutation $\beta_2$702* greatly induced the m24 epitope. Basal expression of the KIM127 epitope on wild-type $\alpha_L\beta_2$ was higher than that of the m24 epitope, and there appeared to be a moderate increase in the $\beta_2$710* mutant. However, expression of the KIM127 epitope was greatly increased by the $\beta_2$702* mutation (Table II). Expression of the KIM127 epitope on $\alpha_L\beta_2$702* was nearly maximal; *i.e.* comparable with constitutively expressed epitopes such as TS2/4.

mAb CBRM1/5 recognizes an activation epitope in the α_M I-domain near the metal ion-dependent adhesion site (MIDAS) motif (39, 45). Expression of the CBRM1/5 epitope was greatly enhanced by the activating β_2 truncation mutation $\beta_2$702*, whereas the $\beta_2$710* mutation did not significantly increase CBRM1/5 binding compared with wild type (Table III). The $\beta_2$702* mutation also greatly increased mAb KIM127 binding to $\alpha_M\beta_2$ (Table III). Thus, constitutively strong ligand binding by $\alpha_L\beta_2$ and $\alpha_M\beta_2$ containing the truncation mutation $\beta_2$702* correlates with exposure of activation epitopes in the extracellular domain.

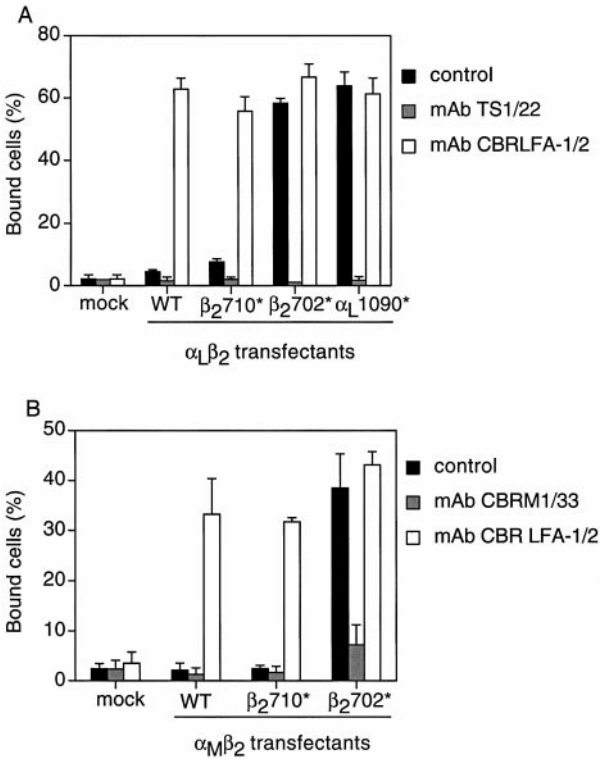

FIG. 3. **Ligand binding activity of $\alpha_L\beta_2$ and $\alpha_M\beta_2$ mutants.** *A,* binding to ICAM-1 of K562 stable transfectants expressing wild-type $\alpha_L\beta_2$ or $\alpha_L\beta_2$ with truncated β_2 ($\beta_2$710* and $\beta_2$702*) or truncated α_L (α_L1090*). ICAM-1 was immobilized in wells of a 96-well plate, and binding of K562 transfectants was determined in the absence (*control*) or presence of the blocking mAb TS1/22 or the activating mAb CBR LFA-1/2 (10 μg/ml). *B,* binding to immobilized iC3b of K562 stable transfectants that express wild-type $\alpha_M\beta_2$ or $\alpha_M\beta_2$ with truncated β_2. iC3b was immobilized, and the binding of K562 transfectants was determined in the absence (*control*) or presence of the blocking mAb CBRM1/33 or the activating mAb CBR LFA-1/2 (10 μg/ml). Results are mean ± S.D. of triplicate samples and are representative of three independent experiments. The expression level of cell surface $\alpha_L\beta_2$ and $\alpha_M\beta_2$ is shown in Fig. 2.

Replacing α_L and β_2 Cytoplasmic Domains with an α-Helical Coiled Coil Constrains $\alpha_L\beta_2$ in the Inactive State—The above results suggest that the membrane proximal region of the β_2 subunit cytoplasmic domains play an important role in the regulation of β_2 integrin function and formation of the $\alpha\beta$ heterodimer. The membrane proximal GFFKR sequence in the α_L cytoplasmic domain regulates α and β heterodimerization and ligand binding by $\alpha_L\beta_2$ (27). We hypothesized, therefore, that the membrane proximal regions of the α and β cytoplasmic domains associate, and such association constrains the integrin in an inactive conformation. To test this hypothesis, we replaced the cytoplasmic domains of α_L and β_2 with a heterodimeric coiled coil. Peptides termed "acid" and "base" were fused to α_L and β_2. These peptides preferentially form heterodimeric as opposed to homodimeric α-helical coiled coils (42). These fusions were termed $\alpha_L$acid and $\beta_2$base, respectively (Fig. 1). As a control, both α_L and β_2 cytoplasmic domains were replaced by the basic peptide ($\alpha_L$base and $\beta_2$base). Dimerization of the two basic peptides is disfavored because of interhelical electrostatic repulsion (42). K562 cell clones were selected that stably expressed $\alpha_L$acid$\beta_2$base and $\alpha_L$base$\beta_2$base at similar levels on the cell surface (Fig. 4).

To test whether the acidic and basic peptide cytoplasmic domains indeed formed an α-helical coiled coil, we examined reactivity with mAb 2H11, which specifically recognizes the acidic and basic peptide heterodimer, and not monomer or homodimer (40). mAb 2H11 immunoprecipitated the $\alpha_L$acid$\beta_2$base complex, but not wild-type $\alpha_L\beta_2$ or $\alpha_L$base$\beta_2$base (Fig. 5). By contrast, mAb TS2/4 to the α_L subunit immunoprecipitated all three types of $\alpha_L\beta_2$ heterodimers (Fig. 5). These results demonstrate that in $\alpha_L$acid$\beta_2$base, the cytoplasmic peptides noncovalently associate to form an α-helical coiled coil.

To test the hypothesis that cytoplasmic association between α_L and β_2 regulates ligand binding, ligand binding activity was compared of $\alpha_L$acid$\beta_2$base, $\alpha_L$base$\beta_2$base, and wild-type $\alpha_L\beta_2$. Clones of K562 stable transfectants that expressed similar levels of surface $\alpha_L\beta_2$ (Fig. 4) were tested for binding to immobilized ICAM-1. Cells that expressed wild-type $\alpha_L\beta_2$ or $\alpha_L$acid$\beta_2$base did not bind to ICAM-1 without activation (Fig. 6*A*). The activating mAb CBR LFA-1/2 or Mn^{2+} greatly increased binding of both wild-type $\alpha_L\beta_2$ and $\alpha_L$acid$\beta_2$base to ICAM-1. By contrast, cells expressing $\alpha_L$base$\beta_2$base strongly bound to ICAM-1 in the absence of activation, and mAb CBR LFA-1/2 or Mn^{2+} did not further increase binding by $\alpha_L$base$\beta_2$base (Fig. 6*A*).

The adhesive function of $\alpha_L$acid$\beta_2$base and $\alpha_L$base$\beta_2$base was further examined in 293T transfectants, in which wild-type $\alpha_L\beta_2$ is basally active (Fig. 6*B*). Wild-type $\alpha_L\beta_2$ and $\alpha_L$base$\beta_2$base in 293T transfectants constitutively bound to ICAM-1, and binding was specific as shown by inhibition with mAb TS1/22 to the α_L I domain. By contrast, $\alpha_L$acid$\beta_2$base showed little binding. However, mAb CBR LFA-1/2 increased ligand binding by $\alpha_L$acid$\beta_2$base to a level comparable with wild-type $\alpha_L\beta_2$ and $\alpha_L$base$\beta_2$base, indicating that lack of ligand binding by $\alpha_L$acid$\beta_2$base was not because of loss of function (Fig. 6*B*).

Constitutive ligand binding by $\alpha_L$base$\beta_2$base correlated with the expression of activation epitopes in the extracellular domain (Table II). Binding of activation-dependent mAbs m24 and KIM127 to $\alpha_L$base$\beta_2$base in K562 cells was greatly increased compared with wild-type $\alpha_L\beta_2$, whereas the level of m24 and KIM127 binding to $\alpha_L$acid$\beta_2$base was similar to wild-type $\alpha_L\beta_2$ (Table II).

DISCUSSION

We have demonstrated that the membrane proximal region of eight residues in the β_2 cytoplasmic domain is required for efficient expression of integrin $\alpha_L\beta_2$ and $\alpha_M\beta_2$ heterodimers on the cell surface and for maintenance of these integrins in the inactive state. Furthermore, we have provided evidence that membrane proximal cytoplasmic association between the α and β subunits constrains an integrin in the inactive state, whereas a lack of association between membrane proximal regions activates an integrin.

It has been shown previously that partial truncations of the β_2 subunit cytoplasmic domain, or mutation of the three contiguous threonines at amino acid residues 736–738 or the phenylalanine at residue 744, abolishes LFA-1 activation by phorbol ester, suggesting that the membrane distal region is involved in the inducible activation of LFA-1 (26, 49). A more recent study showed that complete truncation of the β_2 cytoplasmic domain activated binding through $\alpha_L\beta_2$ of K562 transfectants to ICAM-1 (50). However, the previous studies did not localize the region in the β_2 cytoplasmic domain that maintains LFA-1 in the default inactive state. We have shown that the function of restraining LFA-1 in an inactive state can be localized to a membrane proximal segment with the sequence KALIHLSD. Complete truncation of the β_2 cytoplasmic domain, but not truncation after the KALIHLSD sequence, constitutively activated ligand binding by αLβ2. Furthermore, we generalized this observation to another β_2 integrin, $\alpha_M\beta_2$. Moreover, we demonstrated that the KALIHLSD sequence is required for efficient formation of cell surface $\alpha_L\beta_2$ and $\alpha_M\beta_2$ heterodimers. The membrane proximal sequence in the cytoplasmic domain is conserved among integrin β subunits (50). Deletion of the membrane proximal seven residues in the β_3 cytoplasmic

TABLE II

Expression of activation epitopes by $\alpha_L\beta_2$ mutants

Wild-type and mutant $\alpha_L\beta_2$ were stably expressed in K562 cells, and reactivity of transfectants with mAb m24 and KIM127 was determined by immunofluorescence flow cytometry. Mean fluorescence staining of each antibody after subtraction of the mean fluorescence of the control IgG X63 is expressed as the % mean fluorescence with mAb TS2/4. mAb TS2/4 recognizes the α_L subunit in the $\alpha_L\beta_2$ complex and reacts with wild-type and mutant $\alpha_L\beta_2$ equally well as shown by comparison to many other mAb specific for the α_L and β_2 subunits. Data are mean ± difference from the mean of two independent experiments.

	Binding					
mAb	$\alpha_L\beta_2$	α_L1090*β_2	$\alpha_L\beta_2$710*	$\alpha_L\beta_2$702*	$\alpha_L$acid$\beta_2$base	$\alpha_L$base$\beta_2$base
			(% TS2/4)			
m24	3 ± 1	39 ± 1	7 ± 1	61 ± 2	5 ± 3	48 ± 5
KIM127	19 ± 7	71 ± 0	35 ± 7	92 ± 1	29 ± 5	102 ± 1

TABLE III

Expression of activation epitopes by $\alpha_M\beta_2$ mutants

Reactivity of mAb CBRM1/5 and KIM127 with K562 stable transfectants that express $\alpha_M\beta_2$ with wild-type or truncated β_2 was determined by immunofluorescence flow cytometry. Mean fluorescence staining of each antibody was subtracted by the mean fluorescence of the control IgG X63, and is expressed as % mean fluorescence staining of mAb CBRM1/20, which binds to α_M in the $\alpha_M\beta_2$ complex. mAb CBRM1/20 reacts with wild-type and mutant $\alpha_M\beta_2$ equally well. Data are mean ± difference from the mean of two independent experiments.

	Binding		
mAb	$\alpha_M\beta_2$	$\alpha_M\beta_2$710*	$\alpha_M\beta_2$702*
		(% CBRM1/20)	
CBRM1/5	21 ± 0	30 ± 5	103 ± 4
KIM127	5 ± 1	26 ± 5	64 ± 2

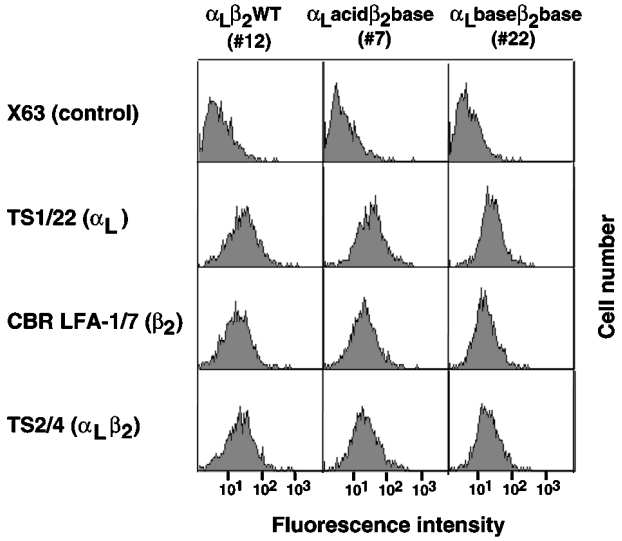

FIG. 4. **Expression of $\alpha_L\beta_2$ coiled coil fusion mutants on the surface of K562 stable transfectants.** K562 cells were stably co-transfected with wild-type α_L and β_2, $\alpha_L$acid and $\beta_2$base, or $\alpha_L$base and $\beta_2$base. Clones of the stable K562 transfectants were stained with the indicated mAbs (shown on the *left*) and analyzed by flow cytometry. Clone numbers are indicated in *parentheses*. *X63*, nonbinding IgG.

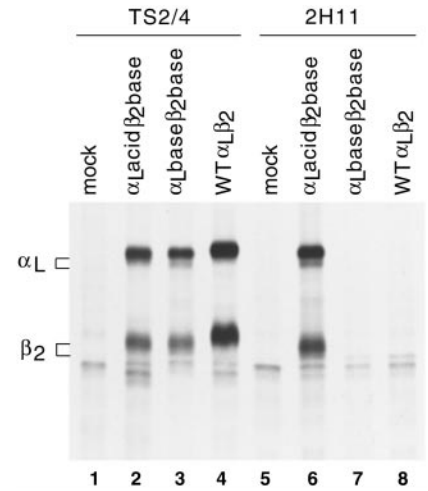

FIG. 5. **The acid and base peptides of $\alpha_L$acid$\beta_2$base associate in an α-helical coiled coil.** Mock-transfected K562 cells or K562 transfectants that stably express wild-type $\alpha_L\beta_2$, $\alpha_L$acid$\beta_2$base or $\alpha_L$base$\beta_2$base were metabolically labeled with [^{35}S]methionine and cysteine. Lysates of the labeled cells were immunoprecipitated with mAb TS2/4 specific for α_L in the $\alpha_L\beta_2$ complex or mAb 2H11 specific for the α-helical coiled coil formed by the acidic and basic peptides. Lysates from equal numbers of labeled cells were subjected to immunoprecipitation, SDS 7.5% polyacrylamide gel electrophoresis, and fluorography. The α_L subunit with the acidic peptide or basic peptide and the β subunit with the basic peptide migrated slightly faster than wild-type α_L and β_2, respectively.

domain results in a constitutively active $\alpha_{IIb}\beta_3$ integrin (30). Similarly, in integrin α subunits, the conserved membrane proximal GFFKR sequence has been shown to be important for regulating integrin $\alpha\beta$ heterodimer assembly and adhesive function (27, 29, 31). Thus, the conserved membrane proximal regions of both integrin α and β cytoplasmic domains control integrin subunit association and ligand binding activity.

The mechanism by which membrane proximal cytoplasmic domain segments regulate integrin activity and efficient association of the α and β subunits has been unclear. Our data strongly support the hypothesis that association between these segments regulates ligand binding activity. We replaced the cytoplasmic domains in $\alpha_L\beta_2$ with peptides that either favor or disfavor association to form an α-helical coiled coil (42). Association of the membrane proximal segments was confirmed by reactivity with mAb 2H11 that recognizes the acid and base peptides only when they are associated with one another in a coiled coil heterodimer (40). We tested the integrin coiled coil fusions in two different cellular contexts, 293T cells and K562 cells, in which wild-type $\alpha_L\beta_2$ is active and inactive, respectively. 293T transfectants that expressed $\alpha_L$acid$\beta_2$base showed little binding to ICAM-1, whereas cells expressing wild-type $\alpha_L\beta_2$ strongly bound to ICAM-1. The activating mAb CBR LFA-1/2 to the β2 subunit or Mn^{2+} could activate ligand binding by $\alpha_L$acid$\beta_2$base, showing that its extracellular domain is competent for activation. Furthermore, $\alpha_L$base$\beta_2$base, in which association of the cytoplasmic basic peptides is disfavored, was constitutively active when expressed in K562 cells in which wild-type $\alpha_L\beta_2$ has little basal activity. These findings provide strong evidence that association of membrane proximal cytoplasmic domains renders an integrin inactive, and that lack of association renders an integrin active (Fig. 1*B*).

Direct association between the membrane proximal GFFKR sequence of the α_{IIb} subunit and the KLLITIHD sequence of the β_3 subunit has been proposed previously based on mutational studies (31). Mutation of Arg-995 in the α_{IIb} GFFKR sequence or Asp-723 in the β_3 KLLITIHD sequence resulted in 39–70% activation of $\alpha_{IIb}\beta_3$, whereas the complementary mutations α_{IIb}

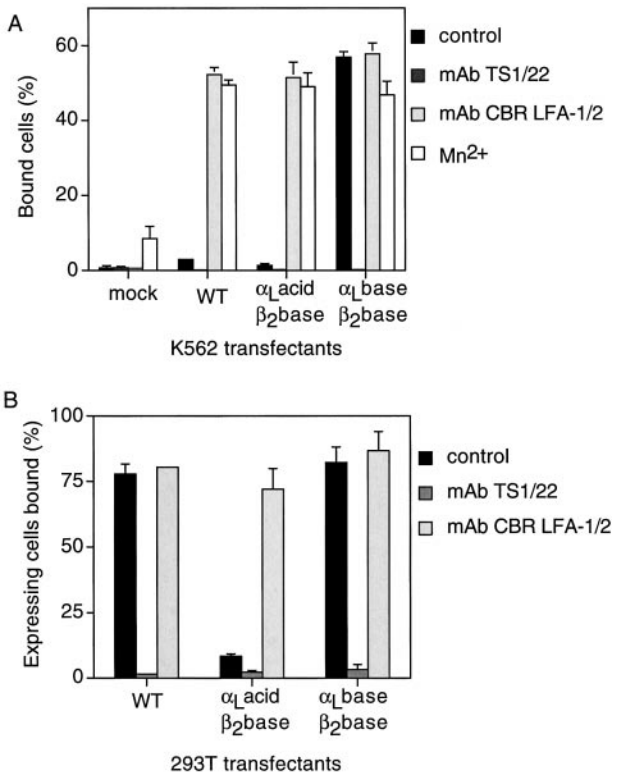

FIG. 6. **Binding of $\alpha_L\beta_2$ coiled coil fusion mutants to purified ICAM-1.** *A,* binding of stable K562 transfectants to ICAM-1. Binding of cells to ICAM-1 was performed in the absence (*control*) or presence of 10 μg/ml of the inhibitory mAb TS1/22 or the activating mAb CBR LFA-1/2 in L15 medium that contains Mg^{2+} and Ca^{2+}. For the Mn^{2+} experiment, the binding assay was conducted in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl supplemented with 1 mM $MnCl_2$. Cell surface expression of $\alpha_L\beta_2$ is shown in Fig. 4. The results are mean ± S.D. of triplicate samples and are representative of three independent experiments. *B,* binding of 293T transient transfectants to ICAM-1. 293T cells were transiently transfected, and binding of the transfectants was determined in the absence (*control*) or presence of the inhibitory mAb TS1/22 or the activating mAb CBR LFA-1/2 at 10 μg/ml. The percent of cells bound to ICAM-1 was divided by the fraction of transiently transfected cells expressing $\alpha_L\beta_2$. The percent of cells expressing $\alpha_L\beta_2$ was 90, 44, and 18% for wild-type $\alpha_L\beta_2$, $\alpha_L$acid$\beta_2$base, and $\alpha_L$base$\beta_2$base transfectants, respectively (determined by flow cytometry with mAb TS2/4 to α_L in the $\alpha_L\beta_2$ complex). The data are mean ± S.D. of triplicate samples and are representative of two independent experiments.

Arg-995→Asp and β_3 Asp-723→Arg resulted in a significantly lower activation of 12% (31). However, no direct evidence for association between the α_{IIb} and β_3 cytoplasmic domains was presented. Moreover, we cannot generalize this result to β_2 integrins, because mutation of the corresponding Asp-709 in the β_2 cytoplasmic domain did not activate ligand binding by $\alpha_L\beta_2$ and $\alpha_M\beta_2$ (3 and 2% of maximal binding, respectively; data not shown). Thus, mechanisms other than a proposed salt bridge between integrin α and β cytoplasmic domains may regulate integrin adhesiveness. Indeed, despite extensive evidence that mutations of the membrane proximal segments of the integrin cytoplasmic domains are activating, there has to date been no direct evidence that association between these segments, either direct or mediated by integrin-associated proteins, regulates adhesiveness. We have demonstrated for the first time that close spatial proximity between membrane proximal α and β subunit segments maintains integrins in an inactive state and that a lack of association results in activation.

The activation state of an integrin is dependent on the cell type in which it is expressed. For example, β_2 integrins in K562 cells require activation to bind to ligands, whereas β_2 integrins are constitutively active in 293T cells, as shown here and previously (25). Presumably, this is due to differential expression of proteins or other factors that modulate integrin function in different cell types. We examined the effect of the $\alpha_L\beta_2$ coiled coil fusions in both types of cellular environments. Interestingly, the effect of the coiled coil mutations was independent of cellular environment. Thus, $\alpha_L$acid$\beta_2$base was inactive in both K562 and 293T cells, whereas $\alpha_L$base$\beta_2$base was active in both cell types. The dominance of the peptides over the cellular environments suggests that the factors that modulate differential integrin activity exert their effect by binding to the cytoplasmic domains of the integrins. Activation of these factors by cytoplasmic signals may regulate binding to integrin cytoplasmic domains and hence the transition between inactive and active wild-type integrins (Fig. 1*B*).

We have demonstrated that activating cytoplasmic domain mutations induce or enhance expression of activation epitopes in $\alpha_L\beta_2$ and $\alpha_M\beta_2$. For $\alpha_L\beta_2$, complete truncation of the β_2 cytoplasmic domain or replacement of the α_L and β_2 cytoplasmic domains with the basic peptides exposed the m24 epitope and enhanced KIM127 epitope expression. Both epitopes localize to the β_2 subunit. The m24 epitope localizes to loops in the I-like domain that are predicted to be near its metal ion-dependent adhesion-like site (47). The KIM127 epitope localizes within cysteine-rich repeat 2, to residues 504, 506, and 508 in the C-terminal region (48). For $\alpha_M\beta_2$, complete truncation of the β_2 cytoplasmic domain greatly increased expression of the CBRM1/5 epitope in the α_M I domain, as well as the KIM127 epitope in β_2. CBRM1/5 binds to the αM I domain very close to the ligand binding site (39, 45). Thus, the activating cytoplasmic domain mutations cause conformational changes in diverse extracellular domains of $\alpha_L\beta_2$ and $\alpha_M\beta_2$. It has previously been shown that complete truncation of the β_2 cytoplasmic domain alters the localization of LFA-1 into clusters, and hence increases cell adhesion (50). Our results suggest that conformational change (affinity regulation), as well as receptor clustering (avidity regulation) play a role in the enhanced adhesiveness of $\alpha_L\beta_2$ and $\alpha_M\beta_2$ induced by activating cytoplasmic domain mutations.

We propose the following model for integrin activation (Fig. 1*B*). The membrane proximal regions of the α and β subunit cytoplasmic domains can associate either directly or indirectly. Under physiological conditions, there is an equilibrium between the association and dissociation of the membrane proximal segments of the cytoplasmic domains that is dynamically regulated. Association constrains the integrin in the inactive state, and dissociation results in activation. Inside-out signaling and integrin binding proteins regulate this equilibrium. Separation of the two membrane proximal regions results in activation. Truncation of the α or β subunit cytoplasmic domain or deletion of the membrane proximal region disrupts this constraint, and activates the integrin. The inactive and active states of an integrin can be mimicked by replacing the integrin cytoplasmic domains with peptides that favor or disfavor, respectively, noncovalent association into a coiled coil. Thus, association of membrane proximal cytoplasmic segments is sufficient to regulate integrin activation and conformational change.

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