Ligand Binding Results in Divalent Cation Displacement from the $\alpha_2\beta_1$ Integrin I Domain: Evidence from Terbium Luminescence Spectroscopy†

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ABSTRACT: The $\alpha_2\beta_1$ integrin serves as a cell surface collagen or collagen/laminin receptor. Binding of the integrin to its ligands is largely mediated by the α_2 subunit I domain and requires the presence of divalent cations. Terbium ion (Tb^{3+}), a fluorescent trivalent cation that often binds divalent cation-binding sites on proteins, supported binding of the I domain to collagen with half-maximal binding occurring at 5.2 ± 1.7 μM Tb^{3+}. By fluorescence resonance energy transfer spectroscopy, Tb^{3+} showed specific and saturable binding to the recombinant I domain with a K_d of 27 ± 4 μM. Although both Mg^{2+} and Mn^{2+} were capable of quenching Tb^{3+} fluorescence, Mn^{2+} was much more effective than Mg^{2+}. The $\alpha_2\beta_1$ integrin also binds the pro-α1(I) collagen carboxyl-terminal propeptide in a Mg^{2+}-dependent manner via the I domain. Recombinant propeptide was used to examine the effect of ligand on the Tb^{3+} binding properties of the α_2 integrin I domain. As propeptide bound to the I domain, Tb^{3+} fluorescence progressively diminished suggesting that as ligand binds to the I domain, either Tb^{3+} is displaced or its fluorescence is quenched. Consistent with the former possibility, little dissociation of collagen-bound I domain occurred upon the addition of EDTA and subsequent incubation. These data support a model in which (1) the divalent cation is required for initial ligand-binding activity of the I domain and (2) ligand binding results in subsequent metal ion displacement to generate a metal-free I domain−ligand complex.

The integrins are a family of cell adhesion molecules that mediate interactions of cells with other cells and with the extracellular matrix. Integrins are heterodimers, consisting of noncovalently associated α and β subunits. They function during development and throughout adulthood in a wide variety of normal and pathologic events. Several α subunits can associate with a given β subunit, or vice versa, leading to the generation of a large number of integrins with varying ligand specificities (for review see ref *1*).

The $\alpha_2\beta_1$ integrin is expressed on a variety of cells including platelets, fibroblasts, lymphocytes, and endothelial and epithelial cells (*2*). The ligand specificity of the $\alpha_2\beta_1$ integrin varies depending upon the cell type on which it is expressed. For instance, $\alpha_2\beta_1$ integrin on platelets binds only collagens, yet when expressed on endothelial and epithelial cells, it binds both collagens and laminin (*3, 4*). Binding of the $\alpha_2\beta_1$ integrin to collagen and $\alpha_2\beta_1$ integrin-mediated adhesion of cells to collagen require the presence of divalent cations. Mg^{2+} and Mn^{2+}, but not Ca^{2+}, support binding and adhesion (*5, 6*). Ca^{2+} not only is incapable of supporting adhesion but also inhibits Mg^{2+}-dependent ligand binding and adhesion (*5, 6*).

The α_2 integrin subunit, like the α_1, α_L, α_X, α_d, α_M, and α_E subunits, contains near its amino terminus an approximately 200 amino acid domain referred to as the I (inserted) domain. Considerable accumulated evidence indicates that the α_2 integrin subunit I domain is directly involved in ligand recognition and binding by the $\alpha_2\beta_1$ integrin. Antiserum prepared against recombinant α_2 I domain disrupts the adhesion of endothelial cells to collagen (*7*). The epitopes recognized by monoclonal antibodies that inhibit the interaction of the integrin with its ligands all map to the α_2 I domain (*8*). Site-directed mutagenesis of critical residues within the I domain disrupts collagen-binding activity of both the I domain and the $\alpha_2\beta_1$ integrin (*8, 9*). Recombinant α_2 integrin I domain binds collagen and laminin in a specific manner (*9*−*12*). I domains from integrin α_1, α_L, and α_M subunits have also been shown to be required for ligand-binding activity of the $\alpha_1\beta_1$, $\alpha_L\beta_2$, and $\alpha_M\beta_2$ integrins, respectively (*13*−*15*).

The pro-α1(I) collagen carboxyl-terminal propeptide, once cleaved from procollagen, can be internalized by the cell and exert feedback inhibition of collagen and fibronectin synthesis (*16*−*18*). Recently, the $\alpha_2\beta_1$ integrin has been shown to bind the procollagen carboxyl-terminal propeptide of type I collagen (*19*). In this report, we show that the α_2 integrin subunit I domain binds the recombinant procollagen carboxyl-terminal propeptide of the α1(I) collagen chain in a divalent cation-dependent manner. In conjunction with the terbium ion (Tb^{3+}), a fluorescent cation that has been used extensively to probe divalent cation-binding sites in proteins including integrins (*20*−*22*), we then used the propeptide as a soluble ligand for the α_2 integrin I domain in a series of resonance energy transfer spectroscopy experiments to

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examine the specificity of the divalent cation-binding site within the α_2 integrin I domain and the effect of the ligand on divalent cation binding. Using this approach, we have obtained evidence that although the divalent cation is required initially for ligand binding by the α_2 integrin I domain, ligand binding results in divalent cation displacement generating a metal-free I domain−ligand complex.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Integrin α_2 I Domain-Containing Proteins. Complementary DNA encoding the human α_2 integrin subunit I domain was generated by PCR[1] using full-length human α_2 integrin cDNA as the template. In addition, cDNA encoding an I domain protein with a 35 residue amino-terminal deletion was also prepared. This protein, which will be referred to as ΔI, lacks the DXSXS portion of the MIDAS motif (*15*). The PCR primers were designed such that both of the amplification products would contain a *Bgl*II restriction site at their 5′ ends and a stop codon followed by an *Xho*I restriction site at their 3′ ends. The PCR products were digested with *Bgl*II and *Xho*I, purified in agarose gels, and cloned into *Bam*HI- and *Xho*I-digested pGEX-5X-1 (Pharmacia Biotech Inc.) for expression as GST fusion proteins (*23*). The I domain PCR product was also cloned into *Bam*HI- and *Sal*I-digested pMAL-c2 for expression as an MBP fusion protein (*24*). The sequence of each insert was determined using the dideoxy chain termination method (*25*) and compared to the published α_2 integrin sequence (*26*). The GST/I domain fusion proteins were expressed in *Escherichia coli* DH5α, purified, and characterized as recently described (*11*). The MBP/I domain fusion protein was purified by affinity chromatography on amylose resin (New England Biolabs, Inc.) as described by the manufacturer.

Cloning and Expression of Pro-α1(I) Collagen Carboxyl-Terminal Propeptide. Complementary DNA encoding the pro-α1(I) collagen carboxyl-terminal propeptide was generated by PCR using plasmid pSTL2 (Dr. Daniel Greenspan, University of Wisconsin) as the template (*27*). This plasmid contains the full-length human α1(I) collagen cDNA. The sequences of forward and reverse primers used in the PCR reaction were 5′-GCCGCTACTCCATGGCTGAT-3′ and 5′-TTTGGGTTGCTCGAGTGTTTCTAGATTGGGG-3′, respectively. These primers were designed such that the amplified DNA product would contain a *Nco*I site at the 5′ end and an *Xba*I site at the 3′ end. The PCR product was digested with *Nco*I and *Xba*I, and the recessed 3′ ends were filled in using Klenow fragment. The resulting blunt-ended product was purified in an agarose gel and cloned into *Sma*I-digested pGEX-5X-1. The insert contains nucleotides 3770−4566 of the published pro-α1(I) collagen mRNA sequence (GenBank accession number Z74615) and encodes amino acids Ala(1218) to Leu(1464). The sequence of the insert was determined using the dideoxy chain termination method (*25*) and compared to the analogous region of the full-length collagen mRNA sequence. The GST-fusion protein was expressed in *E. coli* BL21 and purified as previously described (*11*).

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; GST, glutathione-S-transferase; MBP, maltose-binding protein; MIDAS, metal ion dependent adhesion site; PCR, polymerase chain reaction; TBS, Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4).

Collagen and Pro-α1(I) Collagen Carboxyl-Terminal Propeptide Solid-Phase Binding Assays. Microtiter plates (Immulon 2, Dynatech Laboratories, Inc.) were coated overnight at 4 °C with 0.1 mL of 30 μg/mL type I collagen from calf skin (Sigma) in 0.09% acetic acid or 10 μg/mL purified procollagen carboxyl-terminal propeptide in TBS. Control wells were coated overnight at 4 °C with GST at 10 μg/mL or for 1 h at room temperature with bovine serum albumin (ICN Biomedicals, Inc.) at 300 μg/mL. All wells were washed twice with 0.15 mL of TBS, then blocked for 1 h at room temperature with 0.15 mL of 300 μg/mL bovine serum albumin in TBS. Recombinant proteins were diluted to the desired concentration in various wash buffers (TBS containing 0.05% Tween-20, 30 μg/mL bovine serum albumin, and either 1 mM EDTA, 2 mM $MgCl_2$, or various concentrations of $TbCl_3$). The wells were washed once with 0.15 mL of the appropriate wash buffer, then 0.1 mL of recombinant protein/well was added and allowed to incubate for 1.5 h at room temperature. Wells were then washed three times with 0.15 mL of wash buffer, and 0.1 mL of either a 1:500 dilution of anti-GST antiserum (Pharmacia), a 1:4000 dilution of anti-MBP antiserum (New England Biolabs), or anti-I domain monoclonal antibody 12F1 (Dr. Virgil Woods, University of California at San Diego) at 1 μg/mL in the appropriate wash buffer was added for 1 h at room temperature. Following this incubation, the wells were again washed three times, and then 0.1 mL of a 1:4500 dilution of either pig-anti-goat (for anti-GST), goat-anti-rabbit (for anti-MBP), or goat-anti-mouse (for 12F1) secondary antibody-horseradish peroxidase conjugate (Boehringer Mannheim) in the appropriate wash buffer was added for 1 h at room temperature. The wells were again washed three times, and 0.1 mL of tetramethylbenzidine dihydrochloride (Sigma) prepared according to the manufacturers directions was added per well. After 1 h of substrate conversion, reactions were stopped with 0.025 mL of 4 N H_2SO_4 and the plates read at 450 nm. All binding assays were carried out in triplicate.

Cell Adhesion Assays. Adhesion assays were performed as previously described (*28*). Microtiter plates were coated overnight at 4 °C with type I collagen from calf skin (Sigma) or procollagen carboxyl-terminal propeptide as described above. Platelets were resuspended at 1×10^8/mL and in some cases were preincubated with 10 μg/mL of monoclonal antibodies 6F1 (Dr. Barry Coller, Mt. Sinai Medical Center), 12F1, or C6.7 (*29*−*31*). Following washing of the wells to remove unbound substrate, 0.1 mL of platelets/well was added. Platelets were allowed to adhere for 1 h at room temperature. Nonadherent platelets were removed by washing with TBS containing either 1 mM EDTA or 2 mM $MgCl_2$. Adherent cells were lysed, and the extent of adhesion was determined by assaying for hexoseaminidase (*32*). The reactions were quantitated by reading the plates at 405 nm. All adhesion assays were carried out in triplicate.

Tb^{3+} binding to α_2 Integrin I Domain and Pro-α1(I) Collagen Carboxyl-Terminal Propeptide. The binding of Tb^{3+} to α_2 integrin I domain and procollagen propeptide, either alone or as a mixture, was measured by resonance energy transfer spectroscopy. $TbCl_3$ hexahydrate was purchased from Molecular Probes, Inc. Stock solutions of $TbCl_3$ were prepared at 250 mM and stored at −20 °C. All spectra were measured at 20 °C and at a pH of 7.4 using an Aminco SPF-500 ratio-mode spectrofluorometer. The excitation

wavelength was 285 nm (tryptophan excitation maximum); emission spectra were obtained by scanning from 500 to 600 nm (Tb^{3+} emission range). Since maximum Tb^{3+} fluorescence emission was observed at 545 nm in the resonance energy transfer experiments, Tb^{3+} fluorescence emission was measured at that wavelength. Spectra were obtained using 2 nm band passes for both the excitation and the emission monochromators. The total volume of the sample was not allowed to increase by more than 10% in any of the experiments.

Data for Tb^{3+} binding curves were generated by making successive additions of $TbCl_3$ to cuvettes containing either 2 μM α_2 integrin I domain, 2 μM collagen carboxyl-terminal propeptide, or a mixture of 2 μM I domain and 2 μM propeptide. Following each $TbCl_3$ addition, the sample was mixed and allowed to equilibrate for 5 min prior to obtaining the spectrum. Pilot experiments revealed that equilibrium was achieved during this time. Results are expressed as K_d $\pm$ sem as determined by nonlinear least-squares analysis, as well as by linear fits to Scatchard plots. All binding data were fitted using the equation for a rectangular hyperbola, with noncompetitive terms added where required. Measurements of dynamic light scattering were made at 2 μM I domain in the presence of 1 mM EDTA, 2 mM Mn^{2+}, 27 μM Tb^{3+}, and 70 μM Tb^{3+} using a DynaPro-801 TC Molecular Sizing Instrument.

The ability of divalent cations to quench fluorescence from Tb^{3+} bound to the α_2 integrin I domain was determined by making sequential additions of the indicated divalent cations to cuvettes containing 2 μM I domain and 70 μM Tb^{3+}. Divalent cations (or Na^+ for control) were added successively and spectra were obtained over a range of divalent cation/Tb^{3+} ratios from 1 to 1024. The quenching of fluorescence from Tb^{3+} bound to the α_2 integrin I domain by Mn^{2+} was analyzed by fitting the data to the following equation:

$$F = \frac{F_{max}[Tb^{3+}]}{K_d\left(1 + \frac{[Mn^{2+}]}{K_{i(Tb^{3+})}}\right) + [Tb^{3+}]\left(1 + \frac{[Mn^{2+}]}{K_{i(Mn^{2+})}}\right)} \quad (1)$$

where F_{max} is the fluorescence obtained in the absence of Mn^{2+} and K_d is the dissociation constant of Tb^{3+} for the I domain. For analysis of Mn^{2+} quenching of Tb^{3+} fluorescence, various combinations of both ions were employed. Cuvettes contained 2 μM α_2 integrin I domain and either 50, 66.7, 100, or 200 μM Tb^{3+}. Spectra were obtained at 0, 0.56, 2.2, and 8.9 mM Mn^{2+} for each Tb^{3+} concentration.

To investigate the effect of procollagen propeptide on Tb^{3+} fluorescence from the α_2 integrin I domain, propeptide was added sequentially to a cuvette containing 2 μM α_2 integrin I domain and 100 μM Tb^{3+}. Spectra were obtained over a range of propeptide concentrations from 0.13 to 2.0 μM. Since the propeptide was expressed as a GST fusion protein, the effect of GST on Tb^{3+} fluorescence from the I domain was measured over a similar range of concentrations.

RESULTS

Tb^{3+} Supports α_2 Integrin I Domain Binding to Collagen. Prior to using Tb^{3+} fluorescence to probe the divalent cation-binding site within the α_2 integrin I domain, an analysis of the ability of Tb^{3+} to support the binding of the I domain to collagen was undertaken. As previously demonstrated (*10*, *11*), the α_2 integrin I domain bound collagen in a Mg^{2+}-dependent manner, with no binding to bovine serum albumin (Figure 1A). The α_2 integrin I domain also bound collagen in the presence of Tb^{3+} (Figure 1B). The extent of binding of the I domain to collagen in the presence of 32 μM Tb^{3+} was nearly twice that observed in the presence of 2 mM $MgCl_2$. The binding of I domain to collagen in the presence of Tb^{3+} was saturable, with half-maximal collagen-binding activity occurring at 5.2 $\pm$ 1.7 μM Tb^{3+}. As with Mg^{2+}, little binding of I domain to bovine serum albumin was observed in the presence of Tb^{3+}.

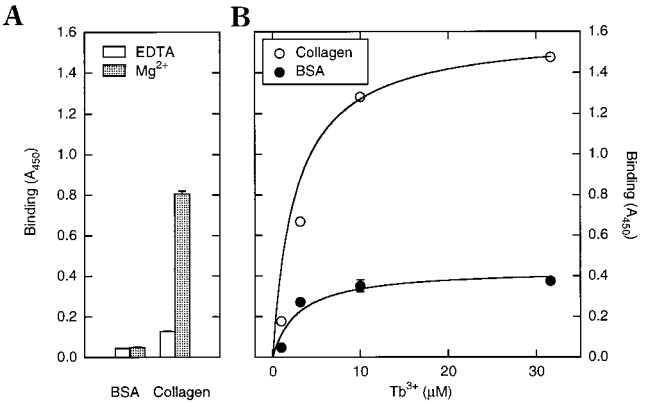

FIGURE 1: Binding of α_2 integrin I domain to collagen. (A) The binding of GST/I domain (25 nM) to type I collagen, or to bovine serum albumin as a control, was measured in a solid-phase binding assay. Binding was determined in the presence of either 1 mM EDTA or 2 mM Mg^{2+}. (B) The binding of GST/I domain (25 nM) to type I collagen, or to bovine serum albumin as a control, was measured over a range of Tb^{3+} concentrations from 1 to 32 μM.

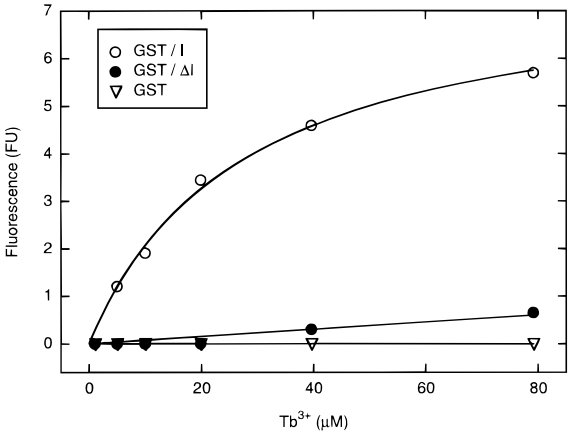

FIGURE 2: Binding of Tb^{3+} to α_2 integrin I domain. The binding of Tb^{3+} to the α_2 integrin I domain was measured by terbium luminescence spectroscopy over a range of Tb^{3+} concentrations from 0 to 80 μM. GST and the ΔI domain fusion (a 35 amino acid amino-terminal deletion mutant that lacks the DXSXS portion of the MIDAS motif) were tested as controls. The concentration of the I domain (or control protein) was 2 μM.

Tb^{3+} Binds to the α_2 Integrin I Domain. The ability of Tb^{3+} to bind the α_2 integrin I domain was investigated further using resonance energy transfer spectroscopy (Figure 2). Tb^{3+} bound to the I domain in a concentration-dependent and saturable manner. The K_d of Tb^{3+} for the I domain was 27 $\pm$ 4 μM. A Scatchard analysis of the Tb^{3+} binding data yielded a straight line and indicated that there are 1.02 Tb^{3+} binding sites/I domain molecule (data not shown). From the Scatchard plot, the K_d of Tb^{3+} for the I domain was 26 $\pm$ 3

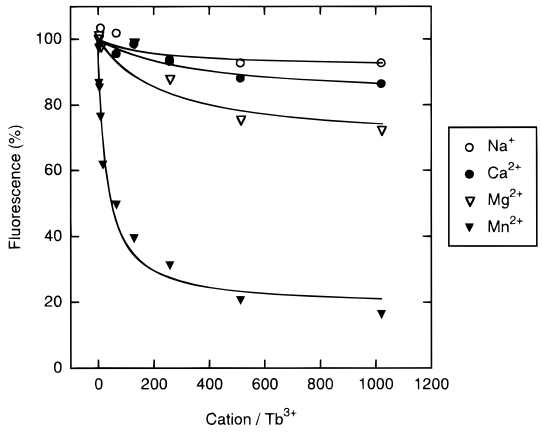

FIGURE 3: Cation-mediated quenching of fluorescence from Tb^{3+} bound to the α_2 integrin I domain. The ability of various cations to quench fluorescence from Tb^{3+} bound to the α_2 integrin I domain was measured by terbium luminescence spectroscopy over a range of cation to Tb^{3+} ratios of 1 to 1024. The concentration of the I domain was 2 μM. The concentration of Tb^{3+} was 70 μM.

μM, in excellent agreement with the above determination. Measurements of dynamic light scattering of the I domain obtained in the presence of EDTA, Mn^{2+}, or Tb^{3+} indicated that no dimerization or aggregation of the I domain occurred under any of these conditions. Tb^{3+} failed to bind either the ΔI protein, a 35 amino acid amino-terminal deletion mutant that lacks the DXSXS portion of the MIDAS motif (*15*, *33*), or GST, which were tested as a negative controls.

Divalent Cation-Mediated Quenching of Fluorescence from Tb^{3+} Bound to the α_2 Integrin I Domain. The ability of various cations to quench Tb^{3+} fluorescence from the α_2 integrin I domain was tested (Figure 3). Cations tested include Mg^{2+} and Mn^{2+}, both of which support the adhesion of cells expressing the $\alpha_2\beta_1$ integrin to collagen (*5*) as well as binding of the α_2 integrin I domain to collagen and laminin (*10*−*12*). Ca^{2+}, which fails to support $\alpha_2\beta_1$ integrin-mediated adhesion of cells to collagen (*5*), adhesion of purified $\alpha_2\beta_1$-bearing liposomes to collagen (*6*), and binding of the recombinant α_2 integrin I domain to collagen (*11*), was also tested. Na^+ was tested as a negative control cation. Mg^{2+} and Mn^{2+} were both capable of quenching fluorescence from Tb^{3+} bound to the I domain; Mn^{2+} was much more effective than Mg^{2+}. The extent of Mn^{2+} quenching of Tb^{3+} fluorescence was sufficient to allow calculation of the K_i for Mn^{2+} by fitting the experimental data to eq 1. This calculation gave 5.2 ± 1.3 mM as the K_i for Mn^{2+} inhibition of Tb^{3+} fluorescence.

Since Mn^{2+} was by far the most effective inhibitor of fluorescence from Tb^{3+} bound to the α_2 integrin I domain, a more extensive analysis was undertaken to determine the mechanism of inhibition. Fluorescence was measured over a range of both Tb^{3+} and Mn^{2+} concentrations, and a linear binding plot was constructed (Figure 4A). The lines obtained by linear regression intersected on the *x*-axis, indicating that Mn^{2+} inhibits fluorescence from Tb^{3+} bound to the I domain by a simple linear noncompetitive mechanism. A replot of the intercepts from the double reciprocal plot versus Mn^{2+} concentration was constructed (Figure 4B). This method gave a K_i of 4.4 ± 0.9 mM for Mn^{2+} inhibition of fluorescence from Tb^{3+} bound to the I domain.

Binding of α_2 Integrin I Domain to the Pro-α1(I) Collagen Carboxyl-Terminal Propeptide. Recently accumulated evidence indicates that type I collagen carboxyl-terminal propeptide exhibits feedback regulation of collagen biosynthesis (*16*, *17*) and that the $\alpha_2\beta_1$ integrin is a cell surface receptor for the propeptide (*19*). Since the α_2 integrin I domain has been shown to contain the ligand-binding site on the $\alpha_2\beta_1$ integrin for collagen (*8*, *10*, *11*) and laminin (*12*), it seemed likely that the I domain may be the site of propeptide binding. To test this hypothesis, propeptide was used as a substrate for platelet adhesion. The results of the platelet adhesion assay are shown in Figure 5A. Platelets adhered to both collagen and the propeptide in the presence of 2 mM Mg^{2+}, but not in the presence of 1 mM EDTA. The $\alpha_2\beta_1$ integrin function-blocking monoclonal antibody 6F1 (*29*) completely blocked platelet adhesion to both substrates. The $\alpha_2\beta_1$ nonfunction-blocking antibody 12F1 (*30*) and an irrelevant control monoclonal antibody to thrombospondin, C6.7 (*31*), had no significant effect on

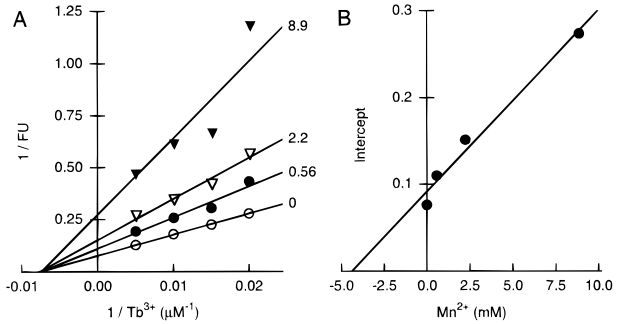

FIGURE 4: Inhibition of fluorescence from Tb^{3+} bound to the α_2 integrin I domain by Mn^{2+}. (A) Linearized plot of Mn^{2+} inhibition of fluorescence from Tb^{3+} bound to the α_2 integrin I domain. Mn^{2+} concentrations tested were 0, 0.56, 2.2, and 8.9 mM. Tb^{3+} concentrations tested were 50, 66.7, 100, and 200 μM. The concentration of the I domain was 2 μM. (FU is fluorescence units.) (B) Replot of the intercepts from (A) versus Mn^{2+} concentration.

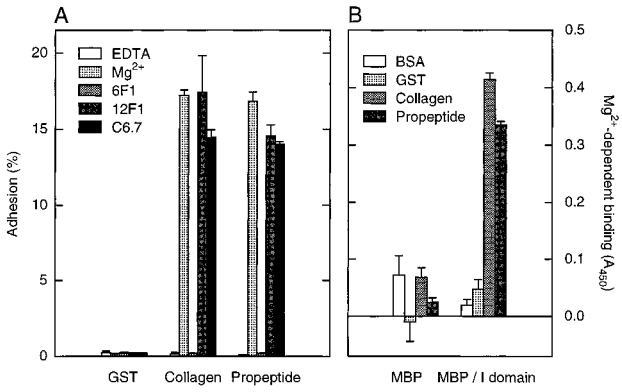

FIGURE 5: (A) Adhesion of platelets to pro-α1(I) collagen carboxyl-terminal propeptide. The adhesion of platelets to pro-α1(I) collagen carboxyl-terminal propeptide was measured in the presence of 1 mM EDTA, 2 mM Mg^{2+} or 2 mM Mg^{2+}, and 10 μg/mL of the indicated monoclonal antibodies. GST and type I collagen were included as negative and positive control substrates, respectively. (B) Binding of the α_2 integrin I domain to pro-α1(I) collagen carboxyl-terminal propeptide. The binding MBP/I domain (200 nM) to pro-α1(I) collagen carboxyl-terminal propeptide was measured in the presence of either 1 mM EDTA or 2 mM Mg^{2+} in a solid-phase binding assay. MBP was tested as a negative control ligand. Bovine serum albumin and GST were tested as negative control substrates; type I collagen was tested as a positive control substrate. The data are expressed as Mg^{2+}-dependent binding (the absorbance obtained in the presence of EDTA was subtracted from that obtained in the presence of Mg^{2+}).

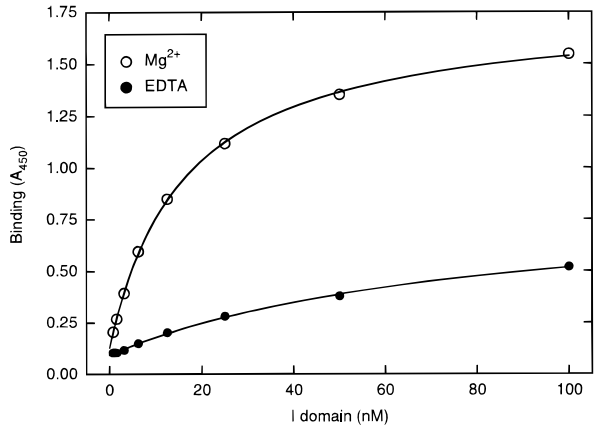

FIGURE 6: Saturable binding of α_2 integrin I domain to pro-α1(I) collagen carboxyl-terminal propeptide. The binding of GST/I domain to pro-α1(I) collagen carboxyl-terminal propeptide binding was measured in a solid-phase binding assay over a range of I domain concentrations from 1 to 100 nM. Assays were performed in the presence of either 1 mM EDTA or 2 mM Mg^{2+}.

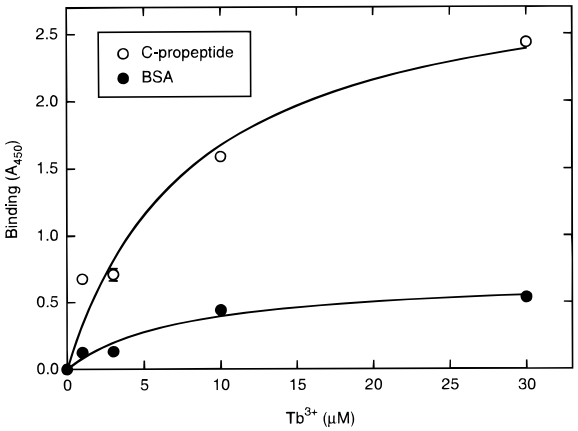

FIGURE 7: Binding of the α_2 integrin I domain to pro-α1(I) collagen carboxyl-terminal propeptide. The binding of GST/I domain (25 nM) to pro-α1(I) collagen carboxyl-terminal propeptide, or to bovine serum albumin as a control, was measured in a solid-phase binding assay over a range of Tb^{3+} concentrations from 0 to 30 μM.

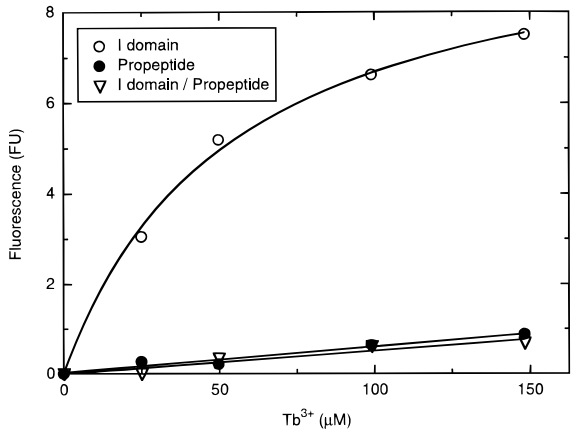

FIGURE 8: Binding of Tb^{3+} to the α_2 integrin I domain, to the pro-α1(I) collagen carboxyl-terminal propeptide, or to the I domain in the presence of the propeptide. The binding of Tb^{3+} to the α_2 integrin I domain, to the pro-α1(I) collagen carboxyl-terminal propeptide, or to the I domain in the presence of an equimolar amount of the propeptide was measured by terbium luminescence spectroscopy over a range of Tb^{3+} concentrations from 0 to 150 μM. Protein concentrations were 2 μM.

platelet adhesion to collagen or to the propeptide. As expected, no platelet adhesion was observed when GST was used as the test substrate. Since the epitope recognized by antibody 6F1 has been mapped to the α_2 I domain (*8*), it seemed likely that the I domain was the site of propeptide binding on the $\alpha_2\beta_1$ integrin. To test this, I domain was examined for propeptide binding in the solid-phase binding assay (Figure 5B). I domain bound both collagen and propeptide in a Mg^{2+}-dependent manner. No Mg^{2+}-dependent binding was observed when bovine serum albumin or GST was tested as a substrate. Since the I domain used in this experiment was an MBP fusion, MBP was also tested for binding. As expected, MBP failed to bind to any of the substrates.

The propeptide-binding activity of the I domain was examined in greater detail over a range of I domain concentrations in both Mg^{2+}- and EDTA-containing buffers (Figure 6). In the presence of Mg^{2+}, I domain bound propeptide in a saturable manner, with half-maximal binding occurring at 16.1 $\pm$ 0.5 nM I domain. Little binding of I domain to propeptide occurred in the presence of EDTA; the signal obtained in the presence of EDTA was approximately one-third of that obtained in the presence of Mg^{2+}. The ability of Tb^{3+} to support propeptide binding of the I domain was also examined (Figure 7). I domain bound propeptide in a Tb^{3+} concentration-dependent manner, with half-maximal propeptide-binding activity occurring at 8.2 $\pm$ 4.3 μM Tb^{3+}, a value similar to that obtained when collagen was used as substrate (Figure 1).

Binding of Tb^{3+} to the α_2 Integrin I Domain in the Presence of the Pro-α1(I) Collagen Carboxyl-Terminal Propeptide. The I domain from the α_M integrin subunit has been crystallized and its crystal structure solved (*15*). The five residues within the α_M subunit I domain that are involved in coordinating Mg^{2+} are completely conserved in the α_2 I domain, suggesting the likelihood that divalent cations are bound in a very similar manner within these two structures. Since the sixth Mg^{2+} coordination site in the α_M crystal is occupied by a carboxylate oxygen from a neighboring I domain molecule within the crystal, it is possible that this site is supplied by a ligand in an actual I domain/ligand complex. Therefore the effect of bound propeptide on Tb^{3+} binding of the I domain was examined. Tb^{3+} binding curves were generated for I domain and the propeptide alone as well as for an equimolar mixture of I domain and the propeptide (Figure 8). In each case, the proteins were present at 2 μM. As was previously shown in Figure 2, Tb^{3+} bound to the I domain in a saturable manner. The propeptide alone failed to bind Tb^{3+}. Surprisingly, no Tb^{3+} fluorescence was observed for the mixture of the I domain and propeptide.

Since the presence of an equimolar amount of propeptide caused almost a complete loss of Tb^{3+} fluorescence from the α_2 integrin I domain, a titration experiment was performed to determine in more detail the effect of propeptide on fluorescence from Tb^{3+} bound to the I domain (Figure 9). Successive additions of propeptide were added to 2 μM I domain. After each addition of propeptide and a five minute equilibration period, emission spectra were obtained for propeptide concentrations from 0.13 to 2.0 μM. As propeptide concentration increased, the fluorescence decreased, with half-maximal inhibition occurring at 0.76 $\pm$ 0.05 μM propeptide. GST was tested as a control and over

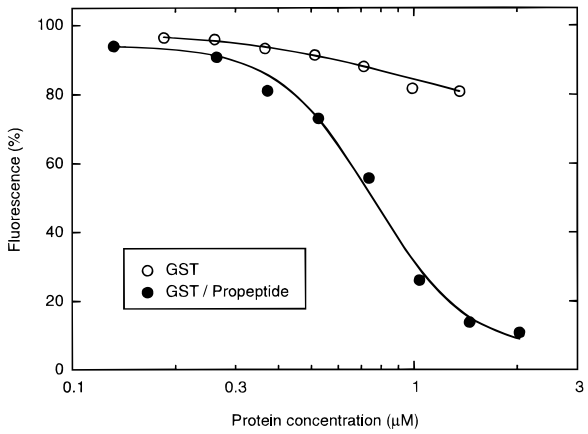

FIGURE 9: Displacement of Tb^{3+} bound to α_2 integrin I domain by pro-α1(I) collagen carboxyl-terminal propeptide. The ability of GST/pro-α1(I) collagen carboxyl-terminal propeptide to displace Tb^{3+} from the α_2 integrin I domain was measured by terbium luminescence spectroscopy over a range of propeptide concentrations from 0.13 to 2.0 μM. The ability of GST to displace Tb^{3+} from the I domain was measured over a similar range of concentrations. The concentration of I domain was 2 μM. The concentration of Tb^{3+} was 100 μM.

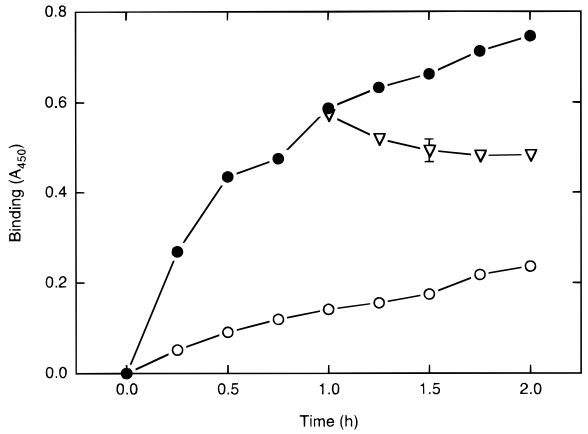

FIGURE 10: Effect of EDTA on collagen-bound α_2 integrin I domain. The binding of I domain (12.5 nM) to type I collagen was measured in the presence of either 1 mM EDTA (open circles) or 2 mM Mg^{2+} (closed circles) in a solid-phase binding assay as a function of time. To assess the ability of EDTA to dissociate the I domain from preformed I domain−collagen complexes, a third set of data (open triangles) was collected by allowing the I domain to bind to collagen in the presence of 2 mM Mg^{2+} for 1 h, at which time EDTA was added to a final concentration of 4 mM for various lengths of time.

a similar range of concentrations had little effect on Tb^{3+} fluorescence from the I domain. These data suggested the possibility that as the ligand bound to I domain, Tb^{3+} was being released from the divalent cation-binding site.

Effect of EDTA on Collagen-Bound α_2 Integrin I Domain. As an alternate approach to support the idea that divalent cation, although required for initiating complex formation, was displaced upon ligand binding, the effect of EDTA on I domain previously bound to collagen in the presence of Mg^{2+} was investigated (Figure 10). I domain binding to collagen was assayed as a function of time, in the presence of either 1 mM EDTA or 2 mM Mg^{2+}. The extent of binding was determined at 15 min intervals over a 2 h period. In addition, several wells contained I domain that was allowed to bind collagen for a period of 1 h, at which time EDTA was added to a final concentration of 4 mM. The effect of the EDTA addition was monitored over an additional 1 h time period with points taken every 15 min. As was expected, the extent of binding of I domain to collagen in the presence of Mg^{2+} continued to increase over the 2 h period, and at any given time point was always 3−4-fold of the binding observed in the presence of EDTA. However, I domain that was allowed to bind collagen in the presence of Mg^{2+} for 1 h prior to the addition of excess EDTA was not released from the collagen by the addition of the EDTA. In fact, even after 1 h in excess EDTA, 85% of the I domain that had bound collagen in the presence of Mg^{2+} remained bound.

DISCUSSION

The adhesion of the cells to the extracellular matrix is in part mediated by the interactions of the $\alpha_2\beta_1$ integrin on the cell surface with collagens and laminins in the matrix. The nature of the interactions of this integrin with its ligands has been extensively studied. The $\alpha_2\beta_1$ integrin-mediated adhesion of cells to collagens requires the presence of divalent cations (*5*). Both Mg^{2+} and Mn^{2+}, as well as several other divalent cations, are capable of supporting the adhesion to collagens of cells expressing the $\alpha_2\beta_1$ integrin. Ca^{2+}, however, is unable to support adhesion and inhibits the Mg^{2+}-dependent collagen-binding activity of cells and liposomes containing purified $\alpha_2\beta_1$ integrin (*6*).

In addition to the requirement for divalent cations, much is known about the location of the ligand-binding site on the $\alpha_2\beta_1$ integrin. An antiserum to the α_2 I domain disrupts adhesion of cells to collagen and laminin (*7*). The epitopes of all $\alpha_2\beta_1$ function-blocking antibodies map to the α_2 integrin subunit I domain (*8*). Point mutations at critical residues in this region of the α_2 subunit abrogate both the adhesion of cells and the binding of recombinant α_2 I domain to collagen (*8*, *9*). Recombinant α_2 I domain specifically binds both collagen and laminin (*9*−*12*).

Like the intact integrin, purified recombinant α_2 integrin I domain binds collagen in the presence of either Mg^{2+} or Mn^{2+}, but not Ca^{2+} (*10*, *11*). Unlike the parent integrin, however, Ca^{2+} does not inhibit the Mg^{2+}-dependent collagen-binding activity of the I domain, suggesting that the other regions of the integrin are required for the inhibitory effect of Ca^{2+} (*11*). A novel divalent cation-binding site was discovered in the α_M I domain when its crystal structure was solved (*33*). This binding site, referred to as a MIDAS motif, consists of five amino acids, some of which are widely separated in the primary sequence. Each of the five residues is involved in the coordination of a magnesium ion. These five amino acids are conserved within the α_2 I domain and all other integrin I domains. Considering the similar requirements for the divalent cation and the high degree of conservation between the α_M and α_2 I domains, it is likely that the α_2 integrin I domain also binds Mg^{2+} via a MIDAS motif. This has been confirmed with the recent publication of the α_2 integrin I domain crystal structure (*34*). However, the coordination of Mg^{2+} by the α_2 integrin I domain differs from the coordination of Mg^{2+} by the α_M and α_L I domains. The former more closely resembles the coordination of Mn^{2+} by the latter two I domains with different amino acid side chains within the MIDAS motif providing coordinating ligands (*34*, *35*).

The ability of the fluorescent cation Tb^{3+} to interact with divalent cation-binding sites on proteins has made it particularly suitable for characterizing the nature and specificity of the cation-binding site present within the α_2 integrin I domain (*20*−*22*). Prior to using Tb^{3+} to assess the divalent cation specificity of the α_2 I domain, an analysis of the ability of Tb^{3+} to support the collagen-binding activity of the I domain was undertaken. Tb^{3+} supported the binding of I domain to collagen, with half-maximal binding activity occurring at 5.2 ± 1.7 μM Tb^{3+}. Only background levels of binding were observed in the absence of Tb^{3+}. Nonspecific binding of I domain to bovine serum albumin in the presence of Tb^{3+} was only about 25% of that observed with collagen as the substrate. Two differences were observed between Mg^{2+}- and Tb^{3+}-supported binding of I domain to collagen. First, the concentration of Tb^{3+} required for half-maximal collagen-binding activity of the I domain was only about 5 μM while approximately 500 μM Mg^{2+} was required for half-maximal collagen-binding activity (*11*). Second, the extent of binding in the presence of only 32 μM Tb^{3+} was twice that observed in the presence of 2 mM Mg^{2+}. These differences may be due to metal ion-dependent differences in conformation that alter the affinity of the I domain for collagen. Several independent lines of evidence support this contention. The recent crystallographic studies suggest that different divalent cations appear to preferentially bind or stabilize I domain conformations of greater or lesser activity (*15*, *34*, *35*). The recently elucidated Mg^{2+}-bound form of the α_2 integrin I domain was thought to represent an inactive or less active form of the integrin (*34*). Our data would suggest that the Tb^{3+}−I domain complex represents an activated conformation. Also, while the α_2 integrin I domain binds collagen to the same extent in either Mg^{2+} or Mn^{2+} (*11*), binding to laminin is considerably greater in the presence of Mn^{2+} as opposed to Mg^{2+} (*12*). The specificity of Tb^{3+} binding to the α_2 I domain was studied by Tb^{3+} luminescence. Tb^{3+} bound to the GST/I domain but not to the ΔI fusion protein, or to GST alone. Since the DXSXS portion of the MIDAS motif is deleted in the ΔI protein, these data provide additional evidence for the presence of a functional MIDAS site within the α_2 I domain and for the specificity of Tb^{3+} binding to the I domain. These results indicate that the same structural features of the I domain participate in the binding of Mg^{2+}, Mn^{2+}, and Tb^{3+}. However, differing metal ion affinities and abilities to support ligand binding require that significant differences in the metal ion−I domain complexes must exist.

Assessment of the ability of several relevant cations to quench fluorescence from Tb^{3+} bound to the α_2 I domain revealed that the divalent cations that are the most capable of supporting binding of the I domain to collagen are also the most efficient at quenching Tb^{3+} fluorescence. Mn^{2+} and Mg^{2+} were both effective at quenching fluorescence from Tb^{3+} bound to the I domain. Neither Ca^{2+} nor the negative control cation Na^{+} had a significant effect on Tb^{3+} fluorescence. Mn^{2+} was much more effective at quenching Tb^{3+} fluorescence than was Mg^{2+}. This was an interesting finding since the same concentration of each (approximately 0.5 mM) is required for half-maximal collagen-binding activity of the I domain (*11*). Taken together, these data reinforce the possibility that ligand binding results from the formation of the active conformation of integrin. We observed in these studies that the concentration dependence of Mn^{2+} that supported ligand binding corresponds very closely with the Mn^{2+} disruption of Tb^{3+}−tryptophan resonance energy transfer.

Kinetic analysis of the inhibition of fluorescence from Tb^{3+} bound to the I domain by Mn^{2+} revealed that Mn^{2+} is a simple linear noncompetitive inhibitor of Tb^{3+} fluorescence. In multireactant systems, mixed-type inhibition can arise in many ways, producing intersecting reciprocal plots. However, the appearance of a simple linear noncompetitive pattern requires that both substrate (Tb^{3+}) and inhibitor (Mn^{2+}) be present in the inactive ESI (I domain−Tb^{3+}−Mn^{2+}) complex (*36*). In this case, inactive means nonfluorescent. Thus a decrease in fluorescence is not due to displacement of Tb^{3+} by Mn^{2+} but rather to the nonfluorescence of the I domain−Tb^{3+}−Mn^{2+} complex. Tb^{3+} binding studies indicate that there is a single Tb^{3+}-binding site on the I domain and that an intact MIDAS motif is required for Tb^{3+} binding. The collagen and propeptide binding studies reveal that Tb^{3+} binds in a physiologically relevant manner and supports ligand binding (both collagen and propeptide) by the I domain. The decrease in Tb^{3+} fluorescence may be due to quenching by Mn^{2+} or to a conformational change in the I domain that results in a shifting of Tb^{3+} away from the tryptophan involved in resonance energy transfer.

There is now crystallographic evidence indicating that the coordination of Mg^{2+} and Mn^{2+} within the MIDAS site of the α_M I domain involves different arrangements of bonds and side chains (*35*). The different mechanisms of metal ion coordination result in alternate positioning of structural elements surrounding the MIDAS motif. Thus, the conformation of the I domain crystallized in the presence of Mg^{2+} was different than that obtained in the presence of Mn^{2+}. We propose that the α_2 I domain MIDAS motif participates in metal binding that promotes the active ligand binding conformation of the I domain. When the metal ion is Tb^{3+}, this active conformation places Tb^{3+} in proximity to a tryptophan resulting in the resonance energy transfer fluorescence that reflects the active conformation of the I domain. Addition of Mg^{2+} or Mn^{2+}, alternate metal ions capable of supporting ligand binding to the I domain, disrupts the resonance energy transfer. Kinetic analysis of the fluorescence inhibition by Mn^{2+} yielded the surprising result that it was noncompetitive inhibition requiring that both Tb^{3+} and Mn^{2+} be in a nonfluorescent although potentially still active conformation of the I domain. This suggested to us the interesting possibility that perhaps these metal ions do not serve a bridging function in ligand binding but rather promote the stabilization of an active conformation of the I domain. One obvious potential consequence of this model would be that nonbridging metal ions could be displaced by ligand binding.

The recent identification of the procollagen carboxyl-terminal propeptide as a ligand for the $\alpha_2\beta_1$ integrin raises the possibility that the propeptide might serve as a soluble model ligand for the α_2 integrin I domain to study integrin−metal−ligand interactions in the Tb^{3+} luminescence assay. Preliminary experiments revealed that an $\alpha_2\beta_1$ integrin recognition site is present within the recombinant α1(I) collagen chain propeptide. Platelets adhered to surfaces coated with the α1(I) propeptide in a divalent cation-dependent manner that was completely inhibited by the $\alpha_2\beta_1$

integrin function-blocking monoclonal antibody 6F1. Furthermore, isolated α_2 I domain bound solid-phase propeptide in a divalent cation-dependent manner. A more detailed analysis of the binding of the α_2 I domain to the propeptide revealed that half-maximal binding of I domain to propeptide required about 16.1 ± 0.5 nM I domain and confirmed the divalent cation requirement of the interaction over a range of I domain concentrations. The Tb^{3+} requirement for half-maximal binding of I domain to propeptide in the solid-phase binding assay, 8.2 ± 4.3 μM, was similar to the Tb^{3+} requirement for binding of the I domain to collagen (5.2 ± 1.7 μM). Thus Tb^{3+} successfully substitutes for Mg^{2+} in the binding of the α_2 I domain to the propeptide.

Analysis of the crystal structures of the α_M, α_L, and α_2 subunit I domains revealed that five of the six coordination ligands involved in Mg^{2+} binding are either hydroxyl oxygen atoms of serines or threonines, or water molecules that form bridges between the Mg^{2+} and aspartates present within the I domain (*15*, *34*, *35*, *37*). In the crystal structure of the α_M I domain, the sixth and apical coordination site was occupied by a carboxylate oxygen from a glutamate residue in a neighboring I domain molecule. This raises the possibility that the physiologically relevant sixth coordination site might be provided by an acidic residue present within the ligand molecule. If this were the case, then inclusion of a soluble ligand for the α_2 I domain in the Tb^{3+} luminescence assay would result in the formation of a better metal ion-binding site and consequent increased affinity of Tb^{3+} for the I domain/ligand complex as compared to the I domain alone. Thus in this model, the presence of ligand would result in a shift to the left of the Tb^{3+} binding curve of the I domain. Upon testing this theory, however, rather than an increase in Tb^{3+} binding to the I domain in the presence of the propeptide relative to the I domain alone, we observed a complete loss of Tb^{3+} fluorescence.

A titration experiment was performed to gain a more detailed understanding of the ability of propeptide to inhibit Tb^{3+} fluorescence from the I domain. As the concentration of propeptide was increased, the level of Tb^{3+} fluorescence decreased in a manner dependent upon the propeptide concentration. These results suggest a model for ligand binding to the α_2 integrin I domain. Divalent cation is required for initial contact of the ligand with the I domain, and an intermediate ternary complex is formed containing the I domain, divalent cation, and ligand. As the binding event progresses, the divalent cation is displaced from the I domain and a metal ion-free I domain/ligand complex is formed.

To test the divalent cation displacement model, the effect of chelating the Mg^{2+} from wells containing preformed collagen-α_2 I domain complexes was examined. If Mg^{2+} were required in a ternary I domain−Mg^{2+}−collagen complex, then the addition of excess EDTA would be expected to cause dissociation of the I domain from collagen. However, if Mg^{2+} were displaced from the I domain upon collagen binding, the addition of EDTA should have little effect. When I domain was allowed to bind collagen for an hour in the presence of Mg^{2+}, and excess EDTA was added for an additional hour, 85% of the I domain that bound collagen in the presence of Mg^{2+} remained bound after an additional hour in the presence of EDTA. It is noteworthy that this experiment provides independent data to support the model of ligand binding-induced divalent cation displacement from the α_2 integrin I domain using the more physiologically relevant ligand, collagen, and the physiologically relevant cation, Mg^{2+}. In a recent study involving the α_1 and α_2 integrin I domains examining only very early times (seconds) following binding using the alternative BIAcore technology, a much greater reversibility of ligand binding upon the addition of EDTA was observed (*38*). It is likely that these results reflect dissociation of ligand from the metal-containing ternary complex. In the longer times conducted in our experiments reversibility was not observed. These findings are entirely consistent with the model proposed above.

An alternative interpretation of the data presented above is the possibility that upon ligand binding, a conformational change occurs in the I domain that causes the distance between the relevant intrinsic fluorophor(s) present within the I domain and the terbium ion to become too great for resonance energy transfer. This is unlikely for the following reasons: (1) Due to the close proximity of trp(188) to the DXSXS portion of the MIDAS motif, it is likely that this tryptophan is the major contributor of resonance energy transfer. While it is possible that a portion of the resonance energy transfer is due a tyrosine residue at position 186, it is unlikely that this residue is a significant contributor considering the higher fluorescence yield of tryptophan and the wavelength of excitation. Regardless, since these residues are within four residues of the DXSXS portion of the MIDAS motif, it is unlikely that any conformational change could cause their distance from the terbium ion to become too great for resonance energy transfer. (2) Consistent with the model, we have failed to observe any change in intrinsic tryptophan fluorescence suggesting that the environment of the tryptophan at position 188 is not altered during ligand binding (unpublished observations). (3) In addition, further evidence supporting the divalent cation displacement model was obtained using an independent experimental system and the more physiologically relevant ligand/cation pair, collagen and Mg^{2+}.

The divalent cation displacement model of ligand binding may not be restricted to I domain-containing integrins. A similar mechanism of divalent cation displacement upon ligand binding to the $\alpha_{IIb}\beta_3$ integrin, an integrin that does not contain an I domain, has been proposed (*22*). The binding of RGD-containing peptides to the $\alpha_{IIb}\beta_3$ integrin or to a synthetic peptide based on a portion of the β_3 integrin subunit resulted in Mn^{2+} displacement from both the integrin and the peptide. Thus the displacement of divalent cations upon ligand binding may be a universal event related to the recognition and binding of ligands by integrins regardless of whether they contain an I domain.

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