Contributions of the I and EF Hand Domains to the Divalent Cation-dependent Collagen Binding Activity of the α2β1 Integrin*

S. Kent Dickeson‡, John J. Walsh, and Samuel A. Santoro§

From the Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110

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The α2β1 integrin binds collagen in a Mg2+-dependent manner that is inhibited by Ca2+. Like the intact integrin, purified recombinant proteins containing the α2 integrin I domain, either alone or with variable numbers of α2 integrin EF hand metal binding sites, bound collagen in a Mg2+-dependent manner, and Ca2+ did not support binding. However, unlike the intact integrin, Ca2+ did not inhibit the Mg2+-dependent binding of any of the fusion proteins to collagen. Binding to collagen was saturable and blocked by the α2β1 function blocking antibody 6F1. Deletional analysis demonstrated that residues present within the amino-terminal 35 amino acids contribute to the 6F1 epitope and are required for Mg2+-dependent collagen binding. The results indicate that the I domain contains a Mg2+ binding site that is essential for collagen binding and that the I domain alone is sufficient for collagen binding. Binding is markedly enhanced in a divalent cation-dependent manner by the addition of the first EF hand motif. Mutation of the EF hand to an inactive form completely abrogated the effect. The sites necessary for Ca2+ inhibition are not present within the I domain or the adjacent region containing the three EF hand sites.

The integrins are heterodimeric cell adhesion molecules that mediate cell-cell adhesion and adhesion between cells and the extracellular matrix. They are widely expressed and function throughout development and adulthood in a variety of normal and pathologic processes (for review, see Ref. 1). The α2β1 integrin is expressed on several different cell types, including endothelial and epithelial cells, fibroblasts, lymphocytes, and platelets (2). The ligand specificity of α2β1 varies with cell type. While it serves as a collagen receptor on platelets and fibroblasts, it can serve as both a collagen and a laminin receptor on endothelial and epithelial cells (3, 4).

Cell adhesion to collagen mediated by the α2β1 integrin is dependent upon the presence of divalent cations (5). Mg2+ is, for example, supported the adhesion. Ca2+ could not substitute for Mg2+ and inhibited the Mg2+-dependent adhesion. The adhesion of liposomes containing purified α2 integrin to collagen was also found to depend on the presence of Mg2+ and to be inhibited by Ca2+ (6). The inhibition of Mg2+-dependent adhesion to collagen of liposomes containing the α2β1 integrin occurred via a simple linear noncompetitive mechanism suggesting that Mg2+ and Ca2+ exert their effects by binding to distinct sites on the α2β1 integrin. Further evidence that Mg2+ and Ca2+ bind to distinct sites was obtained when limited proteolytic digestion of α2β1 gave different cleavage patterns depending on which divalent cation was present (7).

Several potential divalent cation binding sites present in the α2 integrin subunit may mediate the distinct effects of Ca2+ and Mg2+. Within the extracellular domain of α2 are three EF hand motifs. These structures were originally described as Ca2+ binding sites in regulatory proteins (8, 9) but have since been shown to be capable of binding other divalent cations. The α2 subunit is a member of a subset of integrin α subunits that contain an approximately 200 amino acid domain located near the amino terminus often referred to as the I (or inserted) domain. Many I domains, including the αM integrin subunit I domain, contain an additional recently described cation binding site, the metal ion-dependent adhesion site (MIDAS)1 motif (10). The α2 I domain also appears to contain a MIDAS motif since all five of the amino acids that contribute to divalent cation coordination in the αM MIDAS motif are conserved in the α2 integrin I domain.

I domains share homology with the collagen-binding A domains of von Willebrand factor and cartilage matrix proteins suggesting that integrin I domains may be important determinants in ligand binding. The α1 subunit has been shown to be involved with the binding of α2β1 to its ligands, collagen, and laminin (11). Likewise, the αM I domain is required for the interaction of the αMβ2 integrin with its ligands, ICAM-1, IC3b, and fibrinogen (10, 12). The α2 I domain also appears to be important in the binding of the integrin α2β1 with its ligands, ICAM-1, and ICAM-3 (13, 14). Similarly, several lines of evidence implicate the involvement of the α2 I domain in ligand binding activity of the α2β1 integrin. First, a polyclonal antiserum directed against a bacterially expressed α2 I domain fusion protein was shown to block the attachment of endothelial cells to gelatin, type I collagen, and laminin (15). Second, a series of human/bovine α2 integrin chimeras was generated and used to map the epitopes recognized by anti-human α2 integrin monoclonal antibodies that were capable of inhibiting the ligand binding activity of the common α2β1 integrin. All of these antibodies mapped to regions within the α2 I domain, revealing the significance of the I domain with regard to collagen recognition (16). Finally, several mutagenesis studies have demonstrated the importance of amino acids within the α2 I domain for ligand binding (16, 17). Recently, recombinant α2 integrin I domain expressed in bacteria as a glutathione S-transferase (GST) fusion protein has been shown to bind specifically to collagen. However, two reports (17, 18) have presented conflicting data as to whether the I domain, like the intact integrin, binds collagen in a divalent cation-dependent manner.

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‡ Recipient of fellowship support from the American Heart Association-Missouri Affiliate.
§ To whom correspondence should be addressed. Tel.: 314-362-8849; Fax: 314-362-3016.

1 The abbreviations used are: MIDAS, metal ion-dependent adhesion site; GST, glutathione S-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay.
To elucidate the roles of the various divalent cation binding sites present within the α2 integrin subunit with regard to the metal ion-dependent function of the intact α2β1 integrin, we have expressed a series of α2 integrin I domain-containing proteins with either none, one, two, or all three of the EF hand sites. Our results indicate an essential role for the I domain and a here-tofore unrecognized role for the first EF hand motif in divalent cation-dependent collagen binding activity.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Integrin α2 I Domain-containing Proteins—Complementary DNAs encoding the human α2 integrin subunit I domain and the I domain with one, two, or all three of the EF hand divalent cation binding sites were generated by PCR using full-length human α2 integrin cDNA as a template. The proteins encoded by this series constructs will be referred to as I, I+1, I+2, and I+123. All four of the proteins in this series begin at Ser-124 and terminate at Met-349, Gly-516, Lys-570, and Ser-620 of the published α2 sequence (19). In addition, cDNAs encoding a shorter I domain protein lacking the 35 amino-terminal amino acids and the analogous I+1 protein were also prepared. These shorter proteins, referred to as ΔI and ΔI+1, begin at Thr-159 and terminate at Met-349 and Gly-516, respectively. Thus these two proteins lack the DXXS domain of the MIDAS motif. The PCR primers were designed such that all of the amplification products would contain a BglII restriction site at their 5‘ ends and a stop codon followed by an XhoI restriction site at their 3‘ ends. The PCR products were digested with BglII and XhoI, purified in agarose gels, and cloned into PumHI and XhoI-digested GST fusion protein expression vector pGEX-5X-1 (Pharmacia Biotech Inc.). The sequences of all cDNAs used in this study were determined using the dyeodeoxy chain termination method (20) and compared with the published α2 integrin sequence (19).

The sequences of the oligonucleotides used for PCR were as follows: I domain forward primer, 5′-GAAGATCTCTGACGATTCGACAATTCT-3′; I domain reverse primer, 5′-CCCGTCTGATGAGTCTTCTCC-3′; I+1 forward primer, 5′-GAAGATCTCTGACGATTCGACAATTCT-3′; I+1 reverse primer, 5′-CCCGTCTGATGAGTCTTCTCC-3′; I+2 forward primer, 5′-CCCGTCTGATGAGTCTTCTCC-3′; I+2 reverse primer, 5′-CCCGTCTGATGAGTCTTCTCC-3′; ΔI forward primer, 5′-GAAGATCTCTGACGATTCGACAATTCT-3′; ΔI reverse primer, 5′-GAAGATCTCTGACGATTCGACAATTCT-3′.

To prepare I+1 protein with a mutated EF hand, pGEX-5X-1+1 was digested with PstI and XhoI, and the 357-base pair PstI-XhoI fragment was purified in an agarose gel. The fragment was cloned into pBlueScript KS, previously digested with the same enzymes. The Kunkel (21) method of site directed mutagenesis was used to create a double mutant, D272K→A and D272K→A. This protein will be referred to as I+1.

The oligonucleotide used for the mutagenesis reaction was antisense primer sequence 5′-CAGCGTCGATCTTCTCC-3′; I+1 reverse primer, 5′-CCCGTCTGATGAGTCTTCTCC-3′; I+2 reverse primer, 5′-CCCGTCTGATGAGTCTTCTCC-3′; ΔI forward primer, 5′-GAAGATCTCTGACGATTCGACAATTCT-3′; ΔI reverse primer, 5′-GAAGATCTCTGACGATTCGACAATTCT-3′. The PCR products were purified in agarose gels, and the 357-base pair PstI-XhoI fragment containing the double mutation was cloned back into the pGEX-5X-1+1 background. Figure 1 shows a schematic diagram of the constructs used in this study.

Trial inductions were performed to determine whether the selected clones could direct expression of appropriately sized GST fusion proteins. E. coli DH5α containing each of the plasmid constructs was grown at 37 °C in 60 ml of 2 × YT media supplemented with 0.2% glucose and 100 μg/ml ampicillin. Uninduced samples were removed from each culture when the A600 reached 0.3–0.4. Isopropylgalactoside was then added to a final concentration of 1 mM, and the cultures were returned to the incubator for 3 h to allow for accumulation of the expressed proteins. Cell lysates from the uninduced and induced samples were analyzed by SDS-PAGE (22) followed by Coomassie Blue staining. All of the constructs directed the expression of recombinant proteins of the expected size. The site of accumulation and degree of solubility was determined for a representative I domain-containing protein using a published cellular fractionation protocol (23). Bacteria harboring the I+125 construct were grown and induced as described above. At the end of the induction period, the sample was fractionated into media, periplasmic, cytoplasmic soluble, membrane, and insoluble fractions. Each fraction was analyzed by SDS-PAGE, followed by Coomassie Blue staining. The bulk of the recombinant protein (95–100%) accumulated in the insoluble fraction. The other I domain-containing proteins accumulated in the insoluble fraction as well.

For the purification of the fusion proteins, the inductions were performed as above except that the culture volume was increased to 500 ml. At the end of the induction period, the cells were recovered by centrifugation at 2600 × g for 10 min. The cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (10 mM Na2HPO4, 1.8 mM KH2PO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4) and stored at −70 °C until needed. Insoluble fractions were prepared and washed using the Triton X-100 detergent procedure described by Marston (24). The recombinant proteins were solubilized in 8 mM urea on ice for 1 h. After removal of urea-insoluble material by centrifugation at 12,100 × g for 20 min, the urea was diluted to 1.33 mM with 25 mM Tris-HCl, pH 8.0, 10 mM EDTA. The proteins were purified by affinity chromatography on glutathione-Sepharose (Pharmacia) according to a published method (25). Following purification, the proteins were dialyzed extensively against TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Protein yields were determined using the BCA protein assay reagent (Pierce). Figure 2 shows a Coomassie Blue stained SDS-PAGE gel containing approximately 5 μg/lane of each of the recombinant proteins used in this study. All recombinant proteins were subjected to gel filtration analysis on a 10 × 300 mm Superose 12 column (Pharmacia) equilibrated with TBS containing 2 mM MgCl2 (Fig. 3).

Collagen and Laminin Binding Assays—The wells of a 96-well microtiter plate (Immulon 2, Dynatech Laboratories, Inc.) were coated overnight at 4 °C with 0.1 ml of 30 μg/ml collagen I from calf skin (Sigma) in 0.09% acetic acid or with 30 μg/ml laminin I (Collagenic Biomedical Products) in TBS. The wells were washed twice with 0.15 ml of TBS and then blocked for 1 h at room temperature with 0.15 ml of 100 μg/ml bovine serum albumin (ICN Biomedicals, Inc.) in TBS. Recombinant proteins were diluted to 400 nM in various wash buffers (TBS containing 0.05% Tween-20, 10 μg/ml bovine serum albumin, and either 1 mM EDTA, 2 mM CaCl2, 2 mM CaCl2 plus 2 mM MgCl2, or 2 mM MgCl2). The wells were washed once with 0.15 ml of the appropriate wash buffer, and then 0.1 ml of each recombinant protein was added and allowed to interact for 1.5 h at room temperature. Wells were then washed three times with 0.15 ml of the appropriate wash buffer, and then 0.1 ml of a 1:500 dilution of anti-GST antiseraum (Pharmacia) 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 pig-anti-goat 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 manufacturer directions was added per well. After 1 h of substrate conversion, reactions were stopped with 0.025 ml of 4 N H2SO4, and the plates were read at 450 nm.

ELISA—I domain-containing proteins were diluted to 10 μg/ml in TBS containing 2 mM MgCl2 and used to coat the wells of a 96-well microtiter plate (Immulon 2, Dynatech). Coating was carried out overnight at 4 °C with 0.1 ml of solution/well. The wells were washed twice with 0.15 ml of TBS containing 2 mM MgCl2 and then blocked for 1 h at room temperature with 0.15 ml of TBS containing 100 μg/ml bovine serum albumin and 2 mM MgCl2. Primary antibodies used include anti-GST antiseraum and anti-human α2 monoclonal antibodies 6F1 and 12F1. The anti-GST antiseraum was diluted 1:2500; the monoclonal antibodies were diluted to 1 μg/ml in wash buffer (TBS containing 0.05% Tween-20, 10 μg/ml bovine serum albumin, and 2 mM MgCl2). Following blocking, the wells were washed once with 0.15 ml of wash buffer, and then 0.1 ml of primary antibody was added and allowed to interact for 1 h at room temperature. The wells were washed three times with 0.15 ml of wash buffer, and then 0.1 ml of secondary antibody-horseradish peroxidase conjugate (pig-anti-goat for anti-GST or goat-anti-mouse for 6F1 and 12F1) diluted 1:4500 in wash buffer was added per well. Substrate was added, and the plates were read as described above.

Antibody Blocking Assay—The wells of the 96-well microtiter plate were coated with collagen and blocked with bovine serum albumin as described above. I + 1 (100 nM) was preincubated with anti-human α2 antibodies 6F1 or 12F1 (300 nM) for 1 h at room temperature in wash buffer (TBS containing 0.05% Tween-20, 10 μg/ml bovine serum albumin, and 2 mM MgCl2). The wells were washed once with 0.15 ml of wash buffer, and then 0.1 ml of 1/1 antibody mixture was added and allowed to interact for 1.5 h at room temperature. Detection of collagen bound I + 1 was carried out as described above. Substrate was added, and the plates were read at 450 nm.

RESULTS

As an approach to assess the contributions of different classes of divalent cation binding sites present within the α2 integrin...
subunit to the collagen binding activity of the α2β1 integrin, the domains were expressed as recombinant GST fusion proteins containing the α2 integrin I domain alone or in combination with one, two, or all three of the EF hand-like motifs (I, I + 1, I + 12, I + 123). In addition, the I domain was modified by deleting 35 amino acids from its amino terminus in two constructs (ΔI1 and ΔI12). The EF hand motif of I + 1 was modified by incorporating two D→A point mutations to render the EF hand motif incapable of metal binding (I + 1*). The recombinant proteins examined in this investigation are presented schematically in Fig. 1.

After purification by affinity chromatography on glutathione-Sepharose, the proteins were subjected to analysis by SDS-PAGE (Fig. 2). The recombinant proteins were further analyzed by gel filtration chromatography. This analysis confirmed the purity of the proteins in agreement with the SDS-PAGE analysis and revealed the lack of protein aggregation with less than 2.6% of the protein running with an apparent size larger than that predicted for the monomeric species. Quantitative analysis of I domain and I1 proteins revealed that 95 and 96%, respectively, of the protein applied to the column eluted in the monomer peaks. Representative profiles for the I, I + 1, ΔI1, and ΔI12 proteins are shown in Fig. 3. Similar chromatographic profiles were obtained for each of the other proteins used in this study (data not shown).

The proteins were then tested for collagen binding activity. To assess the divalent cation specificity, if any, collagen binding assays were conducted in the presence of 2 mM EDTA, 2 mM Ca2+ or 2 mM Mg2+. The ability of Ca2+ to inhibit any Mg2+-dependent collagen binding was assessed by carrying out the binding assay in the presence of 2 mM of both Ca2+ and Mg2+. The results are shown in Fig. 4. GST alone did not bind specifically to collagen under any of the divalent cation conditions. As previously observed with the intact α2β1 integrin, all of the proteins containing an intact I domain bound collagen in a Mg2+-dependent manner. As also observed with the intact integrin, Ca2+ did not support the collagen binding activity of any of the I domain-containing proteins. However, unlike the intact α2β1 integrin, Mg2+-dependent collagen binding activity of the I domain-containing proteins was not inhibited by Ca2+.
Addition of the first EF hand motif onto the I domain appeared to markedly enhance Mg$^{2+}$-dependent collagen binding activity as revealed by an increased extent of binding. This was most apparent in the I$_1$ and I$_{12}$ proteins.

The necessity of an intact I domain for the collagen binding activity of the constructs was examined with two fusion proteins containing truncated I domains in which the amino-terminal 35 residues were deleted (DI and DI$_{11}$). The deleted region contained the DXXSX sequence, a region thought to be critical for the structural integrity of the MIDAS motif (10). As expected and as shown in Fig. 5, both the I and I$_{11}$ constructs bound collagen in a Mg$^{2+}$-dependent manner. However, neither of the truncated constructs (DI and DI$_{11}$) bound collagen. Thus an intact MIDAS motif is required for Mg$^{2+}$-dependent collagen binding activity.

The contribution of the putative metal binding sequences present within the first EF hand motif to the enhanced collagen binding activity of the I$_{11}$ protein relative to the I domain was examined by mutating two aspartate residues essential for metal binding activity of the motif (Asp-272 and Asp-274) to alanines to create the I$_{11*}$ protein. Unlike the wild-type EF hand motif, the mutated EF hand conferred no enhanced collagen binding activity upon the I domain (Fig. 5). The collagen binding activity of I$_{11*}$ was comparable with that of I domain alone. Thus, sequences within the EF hand motif that confer metal binding properties upon the motif, are essential for the enhancement of collagen binding activity.

The enhanced collagen binding activity of the I$_{11}$ construct relative to that of the I domain alone was examined in greater detail and over a range of concentrations (Fig. 6). Both the I and I$_{11}$ proteins bound to collagen in a concentration-dependent and saturable manner. Whereas half-maximal binding of the I domain protein to collagen occurred at 820 nM, the half-maximal binding of the I$_{11}$ construct was observed at 87 nM.

Mg$^{2+}$ concentrations required for half-maximal collagen binding were determined for I domain, I$_1$, I$_{11}$, I$_{12}$, and I$_{1123}$ proteins and found to be 0.54, 0.31, 0.61, and 1.17 mM, respectively (Fig. 7). Mn$^{2+}$ was also shown to support collagen binding of each of these constructs (Fig. 8). Approximately 0.5 mM Mn$^{2+}$ was required for half-maximal collagen binding.

Anti-human $\alpha_2$ integrin monoclonal antibodies 6F1 and 12F1 were tested for their ability to bind the I, DI, I$_{11}$, and I$_{11*}$ fusion proteins. The results are shown in Fig. 9A. All of the proteins were recognized by the anti-GST antiserum to similar extents, indicating that comparable quantities of the proteins were coated onto the microtiter wells. Monoclonal antibodies 6F1 and 12F1 both bound equivalently to the I and I$_{11}$ proteins that contained intact I domains. Neither bound to the DI and DI$_{11}$ proteins containing truncated I domains. Both of the antibodies also effectively recognized the I$_{11*}$, I$_{112}$, and I$_{1123}$ proteins (data not shown). The same patterns of reactivity were observed in the presence of either 2 mM Mg$^{2+}$ or 2 mM EDTA (data not shown). Thus, the two distinct complex, conformation-dependent epitopes recognized by the 6F1 and
12F1 antibodies (26, 27) are retained to comparable extents in the I and I + 1 constructs, suggesting that the conformation of the I domain in these two proteins is intact. Antibody 6F1 which inhibits the binding of the intact \( \alpha_2 \beta_1 \) integrin to collagen (26), also effectively blocked the binding of the I + 1 protein to collagen. 12F1, an antibody that does not inhibit the binding of the intact \( \alpha_2 \beta_1 \) integrin to collagen (28), similarly failed to inhibit the binding of I + 1 protein to collagen although, as shown in Fig. 9A, the 12F1 antibody effectively bound to the I + 1 protein. The effects of the 6F1 and 12F1 antibodies on collagen binding activity of the I + 1 protein are shown in Fig. 9B.

Since the I and I + 1 proteins exhibited rather different collagen binding activities, we next examined the binding of the two proteins to laminin, a second ligand for the \( \alpha_2 \beta_1 \) integrin. When examined in either Mg\(^{2+}\) or Mn\(^{2+}\)-containing buffers, the I and I + 1 proteins bound laminin to comparable extents (Fig. 10). Unlike binding to collagen, however, binding to laminin was markedly enhanced in the presence of Mn\(^{2+}\). Both I and I + 1 proteins were similarly affected.

**DISCUSSION**

The adhesion of cells to collagen via the \( \alpha_2 \beta_1 \) integrin requires the presence of Mg\(^{2+}\) (5). Ca\(^{2+}\) is incapable of supporting \( \alpha_2 \beta_1 \) integrin-mediated adhesion to collagen and inhibits the Mg\(^{2+}\)-dependent adhesion. Liposomes containing purified \( \alpha_2 \beta_1 \) integrin demonstrated identical metal ion dependence (6). Recently, the I domains of several integrins have been shown to be important determinants of ligand binding (10–14). Evidence of the involvement of the \( \alpha_2 \) I domain in collagen recognition includes: (a) an anti-\( \alpha_2 \) antiserum blocks endothelial cell attachment to collagen (15); (b) several \( \alpha_2 \beta_1 \) integrin function blocking antibodies map to the I domain (16); and (c) purified recombinant \( \alpha_2 \) I domain binds specifically to collagen (17, 18). The crystal structure of the I domain of the related \( \alpha_M \) integrin subunit has been solved and found to contain a single novel Mg\(^{2+}\) binding site that involves residues that are widely separated in the primary sequence (10). In addition to the Mg\(^{2+}\)-binding MIDAS motif within the I domain, the \( \alpha_2 \) integrin subunit contains three EF hand-like metal binding sites in close proximity to the I domain. In the present study, we have prepared a series of \( \alpha_2 \) I domain-containing GST fusion proteins with various numbers of EF hand sites for the purpose of establishing the contributions of the divalent cation binding...
sites present within the I and EF hand domains of the $\alpha_2$ integrin subunit to the metal and ligand binding properties of the integrin.

Like the intact integrin, each of the I domain-containing proteins bound collagen in a Mg$^{2+}$-dependent manner. As also observed with the intact integrin, Ca$^{2+}$ could not substitute for Mg$^{2+}$. Unlike the intact integrin, however, Ca$^{2+}$ did not inhibit the Mg$^{2+}$-dependent binding to collagen of any of the I domain-containing proteins. These data suggest that the sites responsible for Ca$^{2+}$ inhibition of Mg$^{2+}$-dependent collagen binding activity are either not present within the I domain or the region containing the three EF hand structures or that their inhibitory effects are not manifested outside of the context of the intact integrin. The finding that isolated I domain binds collagen in a Mg$^{2+}$-dependent manner is in agreement with the recent work of Tuckwell, *et al.* (18) but contrasts with results from Kamata and Takada (17), who found that independently expressed I domain bound collagen in a divalent cation-independent manner. The reason for the discrepancy is not apparent but may represent an adverse consequence of the iodination of the recombinant I domain protein. We have previously observed deleterious effects on $\alpha_2\beta_1$ integrin structure and ligand binding activity as a result of iodination (7). The results of our binding studies using the DI and DI + 1 proteins strongly

**Fig. 8.** Mn$^{2+}$-dependence of binding of I domain constructs to collagen. Binding of the I domain-containing GST fusion proteins to collagen in the presence of the indicated concentrations of Mn$^{2+}$ was determined.

**Fig. 9.** Antibody recognition and blocking. *A,* anti-GST antiserum and anti-human $\alpha_2$ monoclonal antibodies 6F1 and 12F1 were tested by ELISA for reactivity with I domain and I + 1 proteins. *B,* monoclonal antibodies 6F1 and 12F1 were tested for their ability to inhibit the binding of I + 1 to collagen. I + 1 (100 nM) was preincubated with either antibody (300 nM) for 1 h and then tested in the collagen binding assay.
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from 0.3 to 1.2 mM. These values are similar to those observed for half-maximal collagen of I domain, $I_1$. Mn$^{2+}$ revealed when the protein was crystallized in the presence of EDTA, 2 mM Mg$^{2+}$, or 2 mM Mn$^{2+}$. The results are expressed as divalent cation-dependent binding observed in the presence of Mg$^{2+}$ or Mn$^{2+}$. These data, therefore, suggest that in addition to the region of residues 173–259 identified by Kamata, et al. (16) in their study of human/bovine chimeric $\alpha_2$ integrin, an additional determinant present within residues 124–158 makes an important contribution to the apparently complex epitopes recognized by the 6F1 and 12F1 antibodies.

In summary, the results of this investigation indicate, in agreement with other recent studies, that the $\alpha_2$ integrin subunit I domain is sufficient for collagen binding activity. The data obtained in this study indicate that, while there appears to be a Mg$^{2+}$ binding site within the I domain that is critical for collagen binding, the sites responsible for Ca$^{2+}$ inhibition are not present within the I domain or the region containing the three EF hand structures or that additional portions of the intact integrin are also required in conjunction with Ca$^{2+}$ binding to observe the inhibitory effect. These results are consistent with our earlier observation that Mg$^{2+}$ and Ca$^{2+}$ exert their effects by binding to distinct sites on $\alpha_2\beta_1$ (6, 7).

Finally, our studies reveal several differences between the binding of the I and I + 1 proteins to collagen and laminin. Unlike binding to collagen, which was equivalent in the presence of Mg$^{2+}$ or Mn$^{2+}$, binding of both the I and I + 1 proteins to laminin was greatly enhanced in the presence of Mn$^{2+}$. Furthermore, whereas binding to collagen was considerably enhanced by the addition of the first EF hand motif to the I domain, binding to laminin was essentially unaltered. The structural basis underlying these apparent mechanistic differences warrants further exploration.

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