The interaction of platelets with collagen plays an important role in primary hemostasis. Glycoprotein Ia/IIa (GPIa/IIa, integrin α2β1) is a major platelet receptor for collagen. The binding site for collagen has been mapped to the I domain within the α2 subunit (GPIa). In order to assess the role of the α2-I domain structure in GPIa/IIa binding to collagen, a recombinant I domain (amino acids 126–337) was expressed in Escherichia coli. The α2-I protein bound human types I and III collagen in a saturable and divalent cation-dependent manner and was blocked by the α2β1 function blocking antibody 6F1. The α2-I protein inhibited collagen-induced platelet aggregation (IC50 = 600 nM). Unexpectedly, 6F1, an antibody that fails to inhibit platelet aggregation in platelet-rich plasma, blocked the inhibitory effect of the α2-I protein. The α2-I protein was able to prevent platelet adhesion to a collagen surface exposed to flowing blood under low shear stress. Interestingly, it inhibited platelet adhesion to extracellular matrix at high shear stress. These results, taken together, provide firm evidence that GPIa/IIa directly mediates the first contact of platelets with collagen under both stirring and flow conditions.

Platelet adhesion to subendothelium at sites of vascular injury is a critical initial step in hemostasis. Platelets adhere to collagen by two mechanisms: directly by the interaction of platelet membrane proteins with collagen and indirectly via bridging molecules such as von Willebrand factor (vWF)3 that bind to both platelet glycoprotein (GP) Ibα and collagen. This latter interaction is necessary to confer resistance to detachment under high flow and shear conditions. It also stabilizes platelet adhesion via the collagen receptor (1).

GPIa/IIa (integrin α2β1, VLA2, CD49b/29) is member of the integrin family of heterodimeric molecules that mediate both cell-cell adhesion and adhesion between cells and the extracellular matrix (ECM) (2). The integrin α2β1 is found on several different cell types, and its function may vary depending on the particular cell on which it is expressed. While it is a collagen receptor in platelets and fibroblastic cells, it functions as both a collagen and a laminin receptor on endothelial and epithelial cells (3, 4). It also acts as receptor for the human pathogen echovirus-1 (5) and is involved in the migration of tumor cells within collagenous matrices (6).

Integrin α2β1 is a major collagen receptor in platelets (7). Although α2β1-mediated adhesion appears to be an essential primary step in collagen-platelet interactions, it is still not known whether collagen–α2β1 binding alone is sufficient to support platelet adhesion and to induce collagen-dependent platelet activation (8-10). Platelets from patients described as having mild bleeding disorders due to deficient expression of either the α2-integrin (11–14) or GPVI (15) have demonstrated impaired aggregation in vitro in response to collagen. Antibodies against the collagen receptor GPIV (CD36) partly inhibit platelet adhesion to fibrillar collagen under both static and flow conditions (15-17). However, patients who constitutively lack CD36 have shown normal collagen-induced platelet aggregation (18–20). Recently, an additional collagen receptor has been cloned from human platelets (the 65-kDa protein) and expressed as a recombinant protein (21). This receptor protein binds specifically to type I collagen but not to type III collagen.

The integrin α2β1 is composed of a 150-kDa α2 and a 130-kDa β1 subunit (5). Within the α2 subunit, the 290-amino acid I domain shares homology with the A domains of vWF, complement proteins, cartilage matrix protein, and certain other integrins. There is increasing evidence that I (A) domains play an important role in cell-adhesion protein and cell-matrix interactions. Like other I domains, the α2-I domain contains a cation binding site described as a metal ion-dependent adhesion site (MIDAS) motif (22, 23), which may explain why collagen-induced platelet adhesion and activation are dependent on divalent cations.

Several studies have localized the binding site for collagen to the I domain (amino acids 140–349) of the α2 subunit. First, monoclonal antibodies that block α2β1 interaction with collagen recognize epitopes located between amino acids 173 and 259 within the I domain (24). Second, a polyclonal antiserum prepared against recombinant α2-I domain inhibited endothelial cell attachment to collagen and laminin substrates (25). Finally, some groups have expressed the α2-I domain in bacteria as a glutathione S-transferase (GST) or a maltose binding protein fusion product (26–29). Although some conflicting results have been reported, all functional studies of these expressed proteins confirmed that this region contains the binding site for collagen.

To learn more about the collagen-GPIa/IIa-I domain interac-
tion, we have cloned and expressed in bacteria the a2-I domain spanning residues 126–337. The a2-I protein was purified to homogeneity and assessed for its biological and biochemical properties. The a2-I protein bound to various types of collagen and, when incubated with platelet-rich plasma (PRP), inhibited collagen-induced platelet aggregation. Furthermore, although this protein bound to collagen by a mechanism distinct from that of vWF, it inhibited platelet adhesion to both collagen and endothelial matrix under flow conditions.

**EXPERIMENTAL PROCEDURES**

**Monoclonal Antibodies and Collagen—Antibody 6F1 (30) was obtained from B. Collier (Mt. Sinai Medical Center, New York, NY), antibody 12F1 (31) from V. Woods (University of California, San Diego, CA). Acid-soluble human placenta type I and type III collagen was purchased from Worthington (Lakewood, NJ). Acid-soluble human placenta type I and type III collagen were purchased from Sigma.**

**a2-I Domain Protein—Complementary DNA encoding the human a2 integrin subunit I domain (amino acids 126–337) was generated by polymerase chain reaction (PCR) using full-length human a2 cDNA (provided by M. E. Hemler, Dana-Farber Cancer Institute, Boston, MA) as template. Random primers were designed to introduce a BamHI restriction site at the 5’ end (forward primer, 5’-AAGCGGCGATC-GAGTTTGACGCTCTCA-3’) and HindIII restriction site at the 3’ end (reverse primer, 5’-AAGCCCAAGCTTACCAAGTCTGAA-3’). The PCR product was digested with BamHI and HindIII restriction enzymes and inserted into pQE9 (Qiagen, Chatsworth, CA) for expression in Escherichia coli. The recombinant a2-I domain protein was expressed as a histidine fusion protein containing 12 residues at the N terminus from the expression vector (MRGSHHHHHHHHS).**

**Purification of Recombinant Proteins—Recombinant a2-I protein was expressed in E. coli M15(pREP4) (Qiagen, Chatsworth, CA). Bacteria containing the pQEG3-a2-I fragment were cultured overnight at 37 °C in 50 ml of 25 g/liter tryptone, 15 g/liter yeast extract, 5 g/liter NaCl, pH 7.3, containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. The overnight culture was centrifuged at 12,000 g for 15 min and grown to an A600 of 0.8. The culture was adjusted to 1.5 mM IPTG and incubated for 3.5 h at 37 °C. The cells were then harvested and resuspended in 25 ml of lysozyme buffer (50 mM Tris·Cl, 0.1 mM NaCl, 1 mM EDTA, pH 8.0) containing a final concentration of 250 µg/ml lysozyme and allowed to stand for 15 min at 4 °C. The bacterial cells were lysed in the presence of 1.25 mg/ml deoxycholic acid and 70 µg/ml RNase/DNase. The lysate was centrifuged at 45,000 × g for 25 min. The resultant supernatant was collected and passed over a nickel-nitrilotriacetic acid (Ni2+)-charged Sepharose (Amersham Pharmacia Biotech) column equilibrated with 50 mM Tris·HCl, 500 mM NaCl, pH 7.4. The buffer. The a2-I protein was eluted from the column with 500 mM NaCl or 1 M NaCl.**

**Collagen-binding Assay—Preparation of collagen-coated dishes—**

- **Collagen-induced Platelet Aggregation Assay**—Blood was collected from a healthy donor into a tube containing 3.8% sodium citrate. PRP was prepared by centrifuging the blood at 250 × g for 10 min. Platelet aggregation was carried out in siliconized glass cuvettes at 37 °C with increasing concentrations of collagen in the presence of 25 µM trypsin. The platelet aggregation was monitored using a four-channel aggregometer (Bio/Data Corp., Horsham, PA). PRP was diluted to a platelet concentration of 5 × 10^5/ml in TBS and incubated with varying concentrations of a2-I protein at 37 °C for 5 min. Aggregation was initiated by the addition of 5 µg/ml soluble equine tendon collagen (Helena Laboratories, Beaumont, TX). **Preparation of Collagen-coated Dishes—**

- **Preparation of Extracellular Matrix-coated Dishes—**

  **Human umbilical vein endothelial cells were cultured in growth medium 199 (Bio-Whittaker, Walkersville, MD) containing 20% fetal bovine serum (Sumit Mitotic, P. Collins, CO), 2 mM l-glutamine, 60 units/ml penicillin, 50 µg/ml streptomycin (Life Technologies, Inc.), and 100 µg/ml/1000 units/ml mitomycin (Biomedical Technologies Inc., Stoughton, MA), and 100 mg/ml tissue culture grade heparin (Sigma). The cells were grown on sterile 35-mm dishes (Corning, Corning, NY) for 4 days to reach confluence in a humidified 5% CO2 incubator at 37 °C. The cells were detached by incubation for 20 min at room temperature with 5 µg/ml EDTA in Ca2+ and Mg2+-free Hank’s balanced salt solution (Life Technologies, Inc.).**

The extracellular matrix deposited on the dishes was washed repeatedly with phosphate-buffered saline. **Collagen Binding Assay—**A final concentration of either 500 or 10 µg/ml collagen or a 1% solution of bovine serum albumin (BSA) was added to microtiter wells in 65 mM sodium phosphate buffer, pH 7.2, for 90 min at 37 °C. After washing twice with phosphate-buffered saline, pH 7.4, to remove unadsorbed collagen, residual binding sites were blocked by the addition of 3% BSA in TBS-T for 60 min at 37 °C. **Detection of bound proteins** was performed by incubation for 15 min in 0.05 M H2SO4 and the plates were read at 490 nm. Net specific binding was determined by subtracting optical density values from wells coated only with BSA from the total binding values obtained as described above. Collagen-binding assays in the presence of EDTA or divalent cations were carried out with 35S-labeled a2-I protein (500 nm) and EDTA, MgCl2, or CaCl2 (2 mM). After a 60-min incubation, the wells were washed with 0.05% TFA and the coated dishes were subsequently rinsed and incubated with TBS containing 1% BSA for 30 min at 37 °C to block nonspecific interactions.

**Flow Assays—**A parallel plate flow chamber was assembled as described by the manufacturer (Glyocytect, Rockville, MD). Briefly, the collagen-coated dish formed the lower surface of the chamber and a silicone rubber gasket determined the flow path height of 254 µm. The flow chamber was assembled and filled with TBS. A syringe pump (Harvard Apparatus Inc., Holliston, MA) was used to aspirate blood through the flow chamber. Using different gasket thicknesses, flow rates of 0.48 and 0.6 m/min produced a wall shear rate of 150 s−1 and 1500 s−1, respectively. Blood was collected from healthy adult donors in anticoagulant solution containing 3.8% sodium citrate and was then perfused for 3 min, and the coated dish was washed with TBS. Attached platelets were observed with phase contrast objectives and recorded by videomicroscopy. For the inhibition experiments, the collagen or subendothelial matrix surface were incubated with a2-I protein (4 µM) for 60 min at 37 °C. Blood was incubated with a2-I protein (4 µM) for 5 min at 37 °C. All experiments were performed in duplicate on different days.
RESULTS

After induction with IPTG, bacteria transformed with the plasmid pQE9-α₂-I and expressing α₂-I cDNA were lysed. The resultant bacterial lysate was then passed over a Ni²⁺-column and the bound protein eluted with a step imidazole gradient. The final yield of purified protein was 10 mg/liter of bacterial culture. The calculated molecular mass for the sequence between Asp-126 and Gly-337 is 23,207 Da. The 12 additional amino acids from the vector sequence add another 1613 Da, bringing the total estimated molecular mass to 24,820 Da. This is in good agreement with the estimated molecular mass of the purified material of 27,000 Da (Fig. 1, inset) as assessed by SDS-polyacrylamide gel electrophoresis. Purified α₂-I protein was evaluated for its ability to bind to collagen. As shown in Fig. 1, the α₂-I protein bound to calf skin and human type I and III collagen in a concentration-dependent and saturable manner. The half-maximal binding to the three types of collagen occurred at 650 nM (Fig. 1).

It has been reported that the binding of integrin αβ₃ to collagen is a Mg²⁺-dependent process. To confirm the dependence on divalent cation in α₂-I domain/collagen interactions, we examined the extent of collagen binding in the presence of Mg²⁺, Ca²⁺, and EDTA. Since EDTA impaired the interaction of the anti-polyhistidine-HRP monoclonal antibody with the His tag motif, we expressed and purified metabolically labeled ³⁵S-α₂-I protein. Consistent with previous reports, ³⁵S-α₂-I protein binding to collagen type I was greatly reduced by the addition of 2 mM EDTA (Fig. 2). More detailed studies of the divalent cation dependence of the ³⁵S-α₂-I protein/collagen interaction were then undertaken. It was observed that the addition of Mg²⁺ (2 mM) supported binding while Ca²⁺ (2 mM) only supported low levels of interaction. These results confirmed the integrity of the MIDAS motif in the ³⁵S-α₂-I protein.

Inhibition of platelet GPIa/IIa binding to collagen by α₂-I protein was assessed in two ways. As shown in Fig. 3, we studied the effect of α₂-I protein on collagen-induced platelet aggregation (CIPA). α₂-I protein inhibited platelet aggregation in a dose-dependent manner with an IC₅₀ of 600 nM. At a sufficiently high concentration of α₂-I protein (2 μM), CIPA was completely inhibited. We also examined the effect of the α₂-I protein on platelet interactions with collagen or subendothelial matrix under shear stress. α₂-I protein (4 μM) prevented platelet adhesion to collagen fibrils exposed to whole blood at shear rate of 150 s⁻¹ (Fig. 4A). At higher shear rates (1500 s⁻¹), the protein failed to inhibit this interaction (data not shown). However, the α₂-I protein (4 μM) inhibited platelet adhesion to subendothelial matrix at a shear rate of 1500 s⁻¹ (Fig. 4B). These data confirmed the important role of α₂-β₃ in platelet activation (aggregation) and platelet adhesion under flow conditions.

Since vWF binds to collagen via the vWF-A3 domain, which

FIG. 1. Saturable binding of recombinant α₂-I protein to collagen. The purity of recombinant α₂-I protein was verified by SDS-gel electrophoresis (inset). The purified protein was then tested for its ability to bind collagen. Increasing concentrations of the α₂-I protein were incubated with immobilized collagen: calf skin type I (●), human type I (▲), and human type III (●). Bound protein was determined by ELISA as described under “Experimental Procedures.” Error bars represent S.E. of six similar experiments.

FIG. 2. The effect of EDTA and divalent cations on the collagen binding function of α₂-I protein. The total metabolically labeled ³⁵S-α₂-I protein bound to immobilized type I collagen was normalized to 100%. EDTA, Mg²⁺, or Ca²⁺ (2 mM) were then added to the incubation mixture 500 nM of ³⁵S-α₂-I protein in 75 μl of TBS-T. After incubation for 60 min at 37 °C, the amount of bound radiolabeled protein was determined by scintillation counting. BSA did not support binding of ³⁵S-α₂-I protein. Figure shows mean of three independent experiments.
Aggregation was initiated by the addition of 5 with diluted PRP (1:2) for 5 min at 37 °C in an aggregometer cuvette. Activity of 12F1 were tested for their effects on the collagen binding activity of multimeric vWF to immobilized collagen by the anti-human antibody inhibited platelet aggregation in PRP. However, they were able to block the inhibitory effect of the protein in the CIPA assay. As expected, neither 6F1 nor 12F1 represented six determinations.

possesses a similar three-dimensional structure to α2-I domain, we next examined the ability of the α2-I protein to block the binding of multimeric vWF to collagen. Inhibition of the binding of multimeric vWF to immobilized collagen by α2-I and vWF-A3 proteins is shown in Fig. 5. Both I (A) domain proteins compete with vWF for binding to collagen, but with varying affinities. The IC50 for vWF-A3 protein was 1.0 μM while that for the α2-I protein was 10.2 μM.

The anti-human α2 integrin monoclonal antibodies 6F1 and 12F1 were tested for their effects on the collagen binding activity of α2-I protein in two ways. Antibody 6F1, which inhibits the binding of intact α2β1 integrin to collagen (30), also effectively blocked the binding of the α2-I protein to type I collagen (Fig. 6A). Surprisingly, 12F1, an antibody that does not inhibit the binding of the intact integrin α2β1 to collagen (31), inhibited 50% of the binding of the α2-I protein to collagen. We also studied the effect of the monoclonal antibodies on the α2-I protein in the CIPA assay. As expected, neither 6F1 nor 12F1 antibody inhibited platelet aggregation in PRP. However, they were able to block the inhibitory effect of the α2-I protein in the CIPA assay (Fig. 6B).

**DISCUSSION**

Among the platelet membrane proteins proposed as mediators of platelet adhesion to collagen, GPIa/IIa (integrin α2β1) is considered the major collagen receptor. The I domain present in the α2 chain (GPIa) contains the collagen binding site (24–29). To further our knowledge of α2 subunit structure and function, we have expressed a protein encompassing the α2-I domain (amino acids126–337) in E. coli. This recombinant protein 1) can be readily purified in a soluble form from bacteria, 2) binds specifically to collagen, 3) inhibits collagen-induced platelet aggregation, 4) blocks platelet interaction with collagen or ECM under flow conditions, and 5) inhibits the binding of the multimeric vWF to collagen with a lower affinity than the vWF-A3 protein. Furthermore, its collagen binding ability is blocked by the anti-human α2-integrin monoclonal antibodies 6F1 and 12F1.

Other investigators have used different expression systems to produce α2-I domain fragments in E. coli. In their methods, they have used sonication (26, 27, 29) or denaturing agents (28) to solubilize their recombinant proteins. In contrast, our α2-I protein was purified from the cytoplasmic soluble fraction of enzymatically disrupted bacterial membranes by single-step Ni2+-affinity chromatography. Also of note are the further manipulations to which some groups subjected their recombinant proteins, such as biotinylation or iodination, in order to permit subsequent detection. However, in this study, we expressed and purified metabolically labeled α2-I protein. We believe that the solubility of our recombinant protein is related to the short fusion protein (12 amino acids) coded for in the expression vector and that minimal processing of the purified protein results in a superior starting material for biochemical analysis.

Among the four recombinant α2-I domain expressed and characterized to date, distinctions exist among them with respect to the particular sequence selected for expression, purity of the final product, biophysical properties, and the methods used to functionally analyze the recombinant proteins. However, our results agree, in general, with the previously published observations. The half-maximal binding values reported by three of the previous studies has been calculated using ELISA for protein quantitation. Using similar methods, our value of 650 nM for the three types of collagen used is in good agreement with the range of half-maximal binding concentrations reported previously (400 nM to 1.3 μM) (27–29). In addi-
tion, we are the first group to analyze the binding of the isolated α2-I domain to type III collagen. Our results are in agreement with the work of Saelman et al. (33), who demonstrated the interaction of platelets (via GPIa/IIa) with type III collagen. In one of the four previous studies, Kamata et al. (26) reported the binding of 125I-GST-α2-I protein to collagen with a half-maximal binding of 500 nM. In our study, utilizing purified metabolically labeled 35S-α2-I protein, we obtained a half-maximal binding of 200 nM (data not shown).

Our α2-I protein bound to collagen in a similar manner to the intact integrin α2β1. In this respect, the present study extends the observations of three previously published reports that Mg²⁺ sustained, while EDTA or Ca²⁺ inhibited the binding of α2-I protein to collagen. On the other hand, our findings contrast with those of Kamata et al. (26), who reported that recombinant 125I-GST-α2-I domain bound to collagen in a metal-independent manner. It is possible that this discrepancy is an artifactual consequence of the iodination procedure since we could demonstrate the metal dependence using intrinsically labeled 35S-α2-I protein. The results of our binding studies support the conclusion that the MIDAS motif in the I domain plays an important role in the collagen binding activity of the α2-I domain.

It has been reported that collagen failed to induce platelet aggregation on either integrin α2β1-deficient platelets or in the presence of anti-integrin α2β1 antibody. In this study, the α2-I protein effectively inhibited platelet aggregation in a concentration-dependent fashion. Although the IC₅₀ calculated from our data agrees with a report by Depraetere et al. (29), our study is the first to examine the inhibitory effect of the α2-I protein using PRP. This approach has provided an interesting new insight in that the 6F1 antibody blocked the inhibitory capacity of the α2-I protein. As shown in this study, and in agreement with Coller et al. (30) and Kunicki et al. (34), while 6F1 inhibits CIPA with gel-filtered platelets, 6F1 does not prevent platelet aggregation in PRP. It is of interest that washed platelets were used to produce this antibody. Thus, the discordant 6F1 results might be explained by a continued exposure of the collagen-binding site while a conformational

![Graph](image-url)

**FIG. 5.** Inhibition of vWF binding to collagen by I (A) domain-containing proteins. The binding of plasma vWF to immobilized collagen was measured in the presence of purified vWF-A3 and α2-I proteins at the indicated concentrations or the same volume of TBS in the control mixture. 100% is defined as the fraction of added plasma vWF bound with no competing ligand. Figure is representative of two separate experiments.

![Graph](image-url)

**FIG. 6.** Antibody blocking of α2-I protein binding to collagen. A, monoclonal antibodies 6F1 and 12F1 were tested for their ability to inhibit the binding of α2-I protein to type I collagen. Metabolically labeled 35S-α2-I protein (500 nM) was preincubated with either antibody (1.5 μM) for 30 min and then tested in the collagen-binding assay. Total bound was normalized to 100%. B, neither monoclonal antibody 6F1 nor 12F1 inhibited platelet aggregation at concentrations up to 100 μg/ml. However, when a suspension of PRP (1:2) was incubated with antibody 6F1 or 12F1 and α2-I protein (2 μM), the inhibitory effect of the α2-I protein was abolished by over 95%. Figure is representative of four separate experiments.
change occurs in the α2-I domain during platelet preparation. Another possibility is that the epitope recognized by the antibody could be blocked by a plasma component that is removed by gel filtration.

Our study is also one of the first to analyze simultaneously the interaction between GPIa and collagen under flow conditions using a soluble, high affinity receptor fragment as a ligand. It is known that, at high shear stress, binding of plasma vWF to collagen or ECM and subsequent platelet GPIb binding to bound vWF is required for initial platelet adhesion (1, 35). However, we observed that integrin αβ1 was critical for platelet attachment to subendothelial matrix at high shear rates. This observation agreed with a recent study that used a monoclonal antibody against integrin αβ1 and reported a relatively modest inhibition of platelet attachment to ECM (1). Deposited ECM contains vWF, collagen, and other components that may participate in matrix-platelet interactions (36). However, it was surprising to find that endogenous subendothelial vWF and plasma vWF were not sufficient to support platelet adhesion to ECM. Competition between vWF and the α2-I domain for collagen binding sites is unlikely since in this study we demonstrated that they bind to collagen by different mechanisms. One explanation could be that these cells synthesized a small amount of either endogenous vWF or collagen. Therefore, the consequence would be a limited availability of binding sites for the expression, purification, and the biochemical analysis of recombinant α2-I domain can be employed in future studies to analyze the different ligand specificities of the integrin αβ1 expressed in other cell types. The biological properties of this soluble receptor fragment should provide an interesting model system to design agents that can selectively inhibit shear-dependent platelet adhesion to vascular subendothelium. These results strongly suggest that platelet GPIa/IIa is the major collagen receptor and that the isolated α2-I protein is a promising inhibitor of the platelet-collagen interaction.

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