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Identification in Collagen Type I of an Integrin $\alpha_2\beta_1$-binding Site Containing an Essential GER Sequence*

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The collagen type I-derived fragment α_1(I)CB3 is known to recognize the platelet collagen receptor integrin $\alpha_2\beta_1$ as effectively as the parent collagen, although it lacks platelet-aggregatory activity. We have synthesized the fragment as seven overlapping peptides that spontaneously assemble into triple helices. On the basis of their capacity to bind purified $\alpha_2\beta_1$ and the recombinant α_2 A-domain, and their ability to support $\alpha_2\beta_1$-mediated cell adhesion, we identified two peptides, CB3(I)-5 and -6, which contain an $\alpha_2\beta_1$ recognition site. Synthesis of the peptide CB3(I)-5/6, containing the overlap sequence between peptides 5 and 6, allowed us to locate the binding site within the 15-residue sequence, GFP*GERGVEGPP*GPA (where P* represents hydroxyproline), corresponding to residues 502–516 of the collagen type I α_1 chain. The Glu and Arg residues in the GER triplet were found to be essential for recognition since substitution of either residue with Ala caused a loss of α_2 A-domain binding. By contrast, substitution of the Glu in GVE did not reduce binding, but rather enhanced it slightly. We were unable to detect significant recognition of $\alpha_2\beta_1$ by the peptide CB3(I)-2 containing the putative $\alpha_2\beta_1$ recognition sequence DGEA. Peptides CB3(I)-1 to -6, together with peptide CB3(I)-5/6, exhibited good platelet-aggregatory activity, in some cases better than collagen. However, peptide CB3(I)-7 was inactive, suggesting the presence of an inhibitory element that might account for the lack of aggregatory activity of the parent α_1(I)CB3 fragment.

Integrins that recognize collagen can modulate cell behavior, including adhesion and spreading, migration, division, metabolism, and the expression of the differentiated phenotype. These important processes are physiologically relevant to growth and development, wound repair, and angiogenesis and in pathological processes such as thrombosis and tumor metastasis.

Integrin $\alpha_2\beta_1$ is also an important collagen receptor in hemostasis, where it plays an essential role in the arrest of platelets, under conditions of blood flow, on the collagen fiber surface exposed as a consequence of injury (1–6). Subsequent recognition by the platelet receptor Gp[1] VI of GPP*[2] sequences within the collagen triple helix (7) leads to platelet activation and aggregation with formation of a platelet plug, which serves to stem the loss of blood. Activation of platelets by collagen may also be a cause of thrombosis, especially that associated with rupture of the atherosclerotic plaque, which leads to exposure of underlying collagens (8).

Previous fragmentation studies (9) have indicated the presence of a number of integrin $\alpha_2\beta_1$ recognition sites in collagen I, which, with collagen III, represents the main platelet-aggregatory collagen species in the vessel wall and perivascular space (8). In particular, $\alpha_2\beta_1$-mediated platelet adhesion to fragment α_1(I)CB3 derived from the α_1(I) chain of collagen I is as good as to the parent collagen (9, 10). Inhibition studies with short linear (non-helical) peptides led to the conclusion that the sequence DGEA, corresponding to residues 435–438 of the α_1(I) chain, is an $\alpha_2\beta_1$ recognition sequence in α_1(I)CB3 (11). Despite the ability of the fragment to support integrin-mediated adhesion, it largely lacks the capacity to activate platelets (12, 13), indicating that recognition of $\alpha_2\beta_1$ alone may not be sufficient to induce platelet aggregation, in accord with the requirement for a second receptor, Gp VI (7). In order to discover sequences in α_1(I)CB3 recognizing $\alpha_2\beta_1$, and to gain insight into the structural features of collagen controlling its platelet-activatory ability, we have synthesized the fragment as seven overlapping peptides that spontaneously assemble into triple helices. Based on their ability to support $\alpha_2\beta_1$-mediated adhesion of platelets and HT 1080 cells and to bind the purified integrin and the recombinant α_2 A-domain, we have identified a 15-residue $\alpha_2\beta_1$ recognition sequence, corresponding to residues 502–516 of the α_1(I) chain, containing a GER triplet crucial for activity. We were unable to detect activity within the peptide CB3(I)-2 containing the sequence DGEA. Based on the platelet aggregatory activity of the peptides, we have identified a locus that may contain an inhibitory element and possibly explain the lack of aggregatory activity of the parent fragment.

EXPERIMENTAL PROCEDURES

Materials—Monomeric collagen type I, for use in solid-phase assays and cell adhesion studies, was purified from bovine skin, following limited pepsin digestion, as described previously (9, 12). A suspension of

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[1] The abbreviations used are: Gp, glycoprotein; BSA, bovine serum albumin; CB, cyanogen bromide (in collagen fragment nomenclature); Fmoc, 9-fluorenylmethoxycarbonyl; mAb, monoclonal antibody; TBS, Tris-buffered saline solution.

[2] Standard single-letter nomenclature is used to describe peptide sequences, with P* representing hydroxyproline.

bovine tendon collagen type I fibers, dialyzed and diluted using 0.01 M acetic acid (12), was a gift from Ethicon Inc. (Somerville, NJ) and was used as a standard platelet aggregatory agent.

The anti-(human integrin α_2-subunit) mAb 6F1 (14) was a generous gift from Dr. B. S. Coller (Mount Sinai Hospital, New York, NY). Anti-(human integrin $\alpha_2\beta_1$) mAb, clone Gi9, was purchased from The Binding Site Ltd. (Birmingham, United Kingdom (UK)).

Fmoc-amino acids were from Alexis Corp. (Nottingham, UK). Fmoc-Asp(*t*-butyl ester)- pentafluorophenyl ester and Fmoc-(Fmoc-2-hydroxy-4-methylbenzyl)Gly-OH were from Calbiochem-Novabiochem (UK) Ltd. (Nottingham, UK). *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide and 7-aza-1-hydroxybenzotriazole were from PerSeptive Biosystems (Hertford, UK). TentaGel R RAM resin was from Rapp Polymere GmbH (Tubingen, Germany). Other reagents were analytical grade or better.

Adhesion and Aggregation Assays—Platelet adhesion was measured in Immulon 2 96-well plates using ^{51}Cr-labeled gel-filtered human platelets as described (15), employing the conditions of Santoro (16), *i.e.* static adhesion at room temperature in the presence of Tris-HCl, to avoid the formation of platelet aggregates. We have observed in earlier studies (17) that adhesion is largely of single platelets with no obvious evidence of deposition of platelet aggregates. Results are expressed as bound radioactivity as a percentage of the total applied. Assays were undertaken in triplicate, and data are presented as the mean. Adhesion to BSA-coated wells measured concurrently was never more than 1%. When testing mAbs for inhibitory activity, platelets were pre-incubated with antibody for 15 min. Results presented are representative of three replicate experiments.

Platelet aggregation was measured turbidimetrically at 20 °C using citrated human platelet-rich plasma as previously (15). Data are representative of three repeat experiments involving freshly cross-linked peptides.

Adhesion of human fibrosarcoma (HT 1080) cells was measured as described previously (18). Unattached cells were counted electronically and adhesion calculated as the number of adherent cells expressed as a percentage of the total cell count. Assays were undertaken in triplicate and the data expressed as the mean ± S.D. BSA-coated wells were used to determine nonspecific background adhesion. Data presented are representative of three repeat experiments.

Isolation of Integrin $\alpha_2\beta_1$—Integrin $\alpha_2\beta_1$ was purified from solubilized membranes of human platelets by affinity chromatography on collagen-Sepharose as described (19, 20). Homogeneity was established by polyacrylamide gel electrophoresis, and identification as $\alpha_2\beta_1$ was by immunoprecipitation and Western blotting (20). Protein concentration was determined with BCA reagent (Pierce & Warriner (UK) Ltd., Chester, UK) and the purified integrin was biotinylated using a kit (Amersham Life Sciences, Amersham, UK) according to the manufacturer's instructions. The suitability of the biotinylated product for use in solid-phase assays, as described below, has been demonstrated in earlier studies (20).

Integrin $\alpha_2\beta_1$ Binding Assay—The assay procedure has been described in detail elsewhere (19, 20). Briefly, wells of 96-well enzyme-linked immunosorbent assay plates (Nunc Maxisorp) were coated for 2 h at room temperature with 100 μl of a solution of collagen or peptide, 1–10 μg/ml in 0.01 M acetic acid. Wells were then blocked for 1 h with 100 μl of TBS containing 50 mg/ml BSA (Sigma A4503). After three washes with 200 μl of washing buffer (TBS containing 1 mg/ml BSA; Sigma A7638), 100 μl of 0.5 μg/ml biotinylated integrin in adhesion buffer (washing buffer plus either 2 mM $MgCl_2$ and 1 mM $MnCl_2$ or 10 mM Na_2EDTA, as required) was applied to each well and incubated for 2 h at room temperature. Wells were then washed as above and incubated for 30 min with 100 μl of streptavidin-horseradish peroxidase (Amersham Life Sciences) diluted 1:1500 with washing buffer. The wells were again washed three times and bound integrin detected using a 3,3′,5,5′-tetramethylbenzidine-peroxidase substrate system (KPL, Gaithersburg, Maryland) according to the manufacturer's instructions. Absorbance at 450 nm was measured using a Maxline Emax plate reader (Molecular Devices Ltd., Crawley, UK). Mean values from triplicate assays were corrected for a background reading obtained from using BSA-coated wells, *i.e.* wells coated with 0.01 M acetic acid (vehicle) in the absence of substrate and then blocked with BSA. Background absorbance was never more than 10% of that obtained with collagen. Results are given as the mean ± S.D.

Production of Recombinant Integrin α_2 A-domain—Recombinant human α_2 integrin A-domain was produced, as described by Tuckwell *et al.* (21), from the pertinent cDNA generated by reverse transcription-polymerase chain reaction and cloned into pGEX-2T for transformation of *Escherichia coli* strain DH5αF′. Integrin α_2 A-domain-glutathione *S*-transferase fusion protein was isolated from bacterial lysates by affinity chromatography on a glutathione-agarose column. The suitability of the purified protein for solid-phase assays described below has been established in earlier studies (21, 22).

Integrin α_2 A-domain Binding Assay—Binding was assayed essentially as described by Tuckwell *et al.* (21) and Calderwood *et al.* (22). Briefly, Nunc Maxisorp multiwell plates were coated for 1 h at room temperature with collagen or peptides and blocked with BSA (Sigma A4503) as described above. After three washes with TBS containing 1 mM $MgCl_2$ and 1 mg/ml BSA (Sigma A7638), 100 μl of a solution of 50 μg/ml α_2 A-domain fusion protein in the above buffer or one containing 5 mM EDTA in place of Mg^{2+}, was applied to the wells and the plates incubated for 3 h at room temperature. Wells were then washed as before. Bound A-domain was detected by the addition of 100 μl of 10 μg/ml polyclonal rabbit anti-glutathione *S*-transferase in TBS containing 1 mM $MgCl_2$ and 1 mg/ml BSA (Sigma A7638). Plates were incubated for 45 min and then washed three times as above. After adding 100 μl of peroxidase-conjugated goat anti-rabbit IgG (DAKO Ltd., Ely, UK) diluted 1:2000 in TBS (plus Mg^{2+} and BSA), the plates were incubated for another 45 min, then given a final wash as above. Wells were then treated with a 3,3′,5,5′-tetramethylbenzidine-peroxidase substrate system (KPL) and the absorbance at 450 nm read using the Emax plate reader. Assays were undertaken in triplicate and readings corrected for background as above. Results are expressed as the mean ± S.D.

Peptide Synthesis—Peptides were synthesized as C-terminal amides on TentaGel R RAM resin in a PerSeptive Biosystems 9050 Plus Pep-Synthesiser. In general, Fmoc-amino acids (4 eq) were activated with *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (4 eq) in the presence of diisopropylethylamine (8 eq) (23). 7-Aza-1-hydroxybenzotriazole (4 eq) was added when coupling Asn and Gln. Fmoc deprotection was with a mixture of 2% (v/v) piperidine and 2% (v/v) 1,8-diazabicyclo-[5,4,0]undec-7-ene, except with peptides containing Asp when 20% piperidine and 0.1 M 1-hydroxybenzotriazole in dimethylformamide were used to minimize aspartimide formation (24, 25). Peptides containing Asp-Gly sequences are especially prone to give aspartimide (26), and these were made using Fmoc-(Fmoc-2-hydroxy-4-methylbenzyl)-Gly and Fmoc-Asp(*t*-butyl ester)-pentafluorophenyl ester (27). Peptides were released from the resin by treatment with a mixture of trifluoroacetic acid, thioanisole, ethanedithiol, and triisopropylsilane (90:5:2.5:2.5, by volume) for 6 h at room temperature. Peptides were purified by reverse phase high performance liquid chromatography on a column of Vydac 219TP101522 using a linear gradient of 5–45% acetonitrile in water containing 0.1% trifluoroacetic acid. Fractions containing homogeneous product were identified by analytical high performance liquid chromatography on a column of Vydac 219TP54, pooled, and freeze-dried. All peptides were found to be of the correct theoretical mass by mass spectrometry.

Initially, seven overlapping peptides based on the sequence of the bovine collagen fragment α_1(I)CB3, designated CB3(I)-1 to -7, were synthesized (see Fig. 1). Subsequently, a peptide designated CB3(I)-5/6, containing the overlap sequence shared between peptides 5 and 6, and other peptides containing a variant of this sequence, as detailed later, were made. The triple-helical stability of each peptide was assessed by polarimetry as described previously (15, 18).

Cross-linking—Peptides were cross-linked with 3-(2-pyridyldithio)-propionic acid *N*-hydroxysuccinimide ester as before (15, 18).

RESULTS

The sequences of the peptides used in this study are shown in Fig. 1. Additional GPP* triplets were introduced at each end of the sequence to promote the formation of a stable triple-helical structure at 20 °C, the temperature at which assays were made (18). The triple-helical conformation is essential for integrin binding and $\alpha_2\beta_1$-mediated cell adhesion (9, 18, 28). A GPC triplet was also added at the N and C termini to allow cross-linking to produce a polymer, since quaternary as well as tertiary structure is necessary for the expression of platelet aggregatory activity (9, 18, 28). As expected, all of the peptides spontaneously adopted a triple-helical conformation. Melting temperatures ($T_{m(1/2)}$) of the peptides were as follows: CB3(I)-1, 32 °C; CB3(I)-2, 39 °C; CB3(I)-3, 37 °C; CB3(I)-4, 36 °C; CB3(I)-5, 30 °C; CB3(I)-6, 30 °C; CB3(I)-7, 26 °C; CB3(I)-5/6,

CB3(I)-1:
GPC(GPP*)$_3$**GFP*GPKGAAGEP*GKAGERGVP*GPP*GAVGPA**(GPP*)$_3$GPC

CB3(I)-2:
GPC(GPP*)$_3$**GPP*GAVGPAGKDGEAGAQGPP*GPAGPAGER**(GPP*)$_3$GPC

CB3(I)-3:
GPC(GPP*)$_3$**GPAGPAGERGEQGPAGSP*GFQGLP*GPAGPP***(GPP*)$_3$GPC

CB3(I)-4:
GPC(GPP*)$_3$**GLP*GPAGPP*GEAGKP*GEQGVP*GDLGAP*GPS**(GPP*)$_3$GPC

CB3(I)-5:
GPC(GPP*)$_3$**GDLGAP*GPSGARGERGFP*GERGVEGPP*GPA**(GPP*)$_3$GPC

CB3(I)-6:
GPC(GPP*)$_3$**GFP*GERGVEGPP*GPAGPRGANGAP*GNDGAK**(GPP*)$_3$GPC

CB3(I)-7:
GPC(GPP*)$_3$**GANGAP*GNDGAKGDAGAP*GAP*GSQGAP*GLQ**(GPP*)$_3$GPC

CB3(I)-5/6:
GPC(GPP*)$_3$**GFP*GERGVEGPP*GPA**(GPP*)$_3$GPC

desE-5/6:
GPC(GPP*)$_3$**GFP*GARGVAGPP*GPA**(GPP*)$_3$GPC

GVA-5/6:
GPC(GPP*)$_3$**GFP*GERGVAGPP*GPA**(GPP*)$_3$GPC

GAR-5/6:
GPC(GPP*)$_3$**GFP*GARGVEGPP*GPA**(GPP*)$_3$GPC

GEA-5/6:
GPC(GPP*)$_3$**GFP*GEAGVEGPP*GPA**(GPP*)$_3$GPC

FIG. 1. **Peptide sequences.** For peptides CB3(I)-1 to -7, the sequences corresponding to those in α_1(I)CB3 are shown in *bold*. The overlap between adjacent peptides is *underlined*. The seven peptides span the 149-residue length of α_1(I)CB3 except for the final Gly and Met residues. CB3(I)-5/6 contains the overlap sequence between CB3(I)-5 and CB3(I)-6, and is shown in *bold*. The sequences of the variants of CB3(I)-5/6 are also shown.

39 °C. A representative melting curve, of CB3(I)-6, is shown in Fig. 2.

Binding of $\alpha_2\beta_1$—In accord with previous findings (4, 19, 20), we could detect binding of $\alpha_2\beta_1$ to collagen in solid-phase assays, which was largely divalent cation-dependent and strongly inhibited by anti-$\alpha_2\beta_1$ mAbs (Fig. 3, *a–c*). Of the seven peptides CB3(I)-1 to -7, only peptides 5 and 6 showed $\alpha_2\beta_1$ binding (Fig. 3, *a* and *b*). Binding to these two peptides was greater than to collagen (Fig. 3*b*) and strongly inhibited by EDTA (90%; data not shown). Peptide CB3(I)-5/6, containing the overlap sequence between peptides 5 and 6 (see Fig. 1) also exhibited binding of $\alpha_2\beta_1$ comparable to that to collagen. In this case, too, binding was strongly divalent cation-dependent and fully inhibited by anti-α_2 mAb 6F1 (Fig. 3*c*). These results suggested that the binding exhibited by peptides CB3(I)-5 and -6 was attributable to a single binding locus contained in the overlap sequence GFP*GERGVEGPP*GPA.

Binding of α_2 A-domain—As before (20, 21), we observed divalent cation-dependent binding of α_2 A-domain to collagen which could be prevented by anti-$\alpha_2\beta_1$ mAbs (Fig. 4, *a* and *b*). As for intact $\alpha_2\beta_1$, significant cation-dependent binding occurred only to peptides CB3(I)-5, -6, and -5/6 (Fig. 4, *a* and *b*). Slight binding to CB3(I)-3, -4, and -7 was nearly all cation-independent (nonspecific). Binding to CB3(I)-5/6 was consistently greater than to collagen (Fig. 4, *a* and *b*) and was fully inhibited by the anti-$\alpha_2\beta_1$ mAbs 6F1 and Gi9. Inhibition by Gi9 is shown in Fig. 4*b* as an example. Inhibition by 6F1 and Gi9

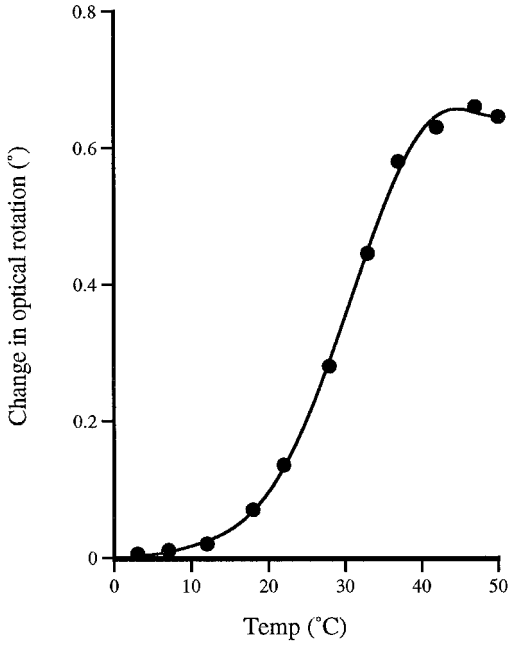

FIG. 2. **Melting curve of peptide CB3(I)-6.**

reflects the known ability of these mAbs to bind to isolated α_2 A-domain (21, 22). These results confirmed the presence of an $\alpha_2\beta_1$ recognition site in the CB3(I)-5/6 sequence.

In order to determine the structural features of this sequence essential for activity, we synthesized a number of modifications of the peptide 5/6, as follows: peptide desE-5/6, in which the two Glu residues are replaced by Ala ($T_{m(1/2)}$ = 44 °C); peptide GVA-5/6, in which the Glu in GVE is replaced by Ala ($T_{m(1/2)}$ = 41 °C); peptide GAR-5/6, in which the Glu in GER is replaced by Ala ($T_{m(1/2)}$ = 42 °C); and peptide GEA-5/6, in which the Arg in GER is replaced by Ala ($T_{m(1/2)}$ = 38 °C).

Binding studies with α_2 A-domain indicated that the Glu and Arg residues in GER were important for the recognition of $\alpha_2\beta_1$, while the Glu residue in GVE was not. Thus, there was excellent binding to peptide GVA-5/6, better than to collagen and peptide CB3(I)-5/6, but poor binding to peptides desE-5/6, GEA-5/6, and GAR-5/6 (Fig. 5).

Adhesion of HT 1080 Cells—Adhesion of these cells to collagen is essentially by a single mechanism involving integrin $\alpha_2\beta_1$ (18–21). In the present study, adhesion was measured at 20 °C to ensure that peptides were fully triple-helical. In accord with our previous data (18), we observed good adhesion of the cells to collagen, which was strongly inhibited by the anti-$\alpha_2\beta_1$ mAb, 6F1 (Fig. 6). No significant adhesion above background occurred to peptides CB3(I)-2, -3, -4, and -7, but peptides CB3(I)-5 and -6 supported adhesion, which was always as good as to collagen and was inhibited equally as well by mAb 6F1 (Fig. 6). Cells remaining attached in the presence of 6F1 showed a complete absence of spreading (not shown). Interestingly, HT 1080 cells consistently showed adhesion to peptide CB3(I)-1, around two-thirds of that to collagen, and this adhesion, too, was inhibited by 6F1 (Fig. 6). In confirmation of the existence of an integrin $\alpha_2\beta_1$ recognition sequence in the overlap between peptides CB3(I)-5 and -6, peptide CB3(I)-5/6 supported adhesion of HT 1080 cells that was as good as to collagen and was just as susceptible to blockade with 6F1 (Fig. 6).

Platelet Adhesion to CB3(I)-1 to -7 and to CB3(I)-5/6—Platelet adhesion to immobilized monomeric collagen is Mg^{2+}-dependent and fully inhibitable by an anti-$\alpha_2\beta_1$ mAb such as 6F1 (4, 9, 10, 14, 16, 29). However, collagen fibers can also support cation-independent platelet adhesion (9, 27). Adhesion to the

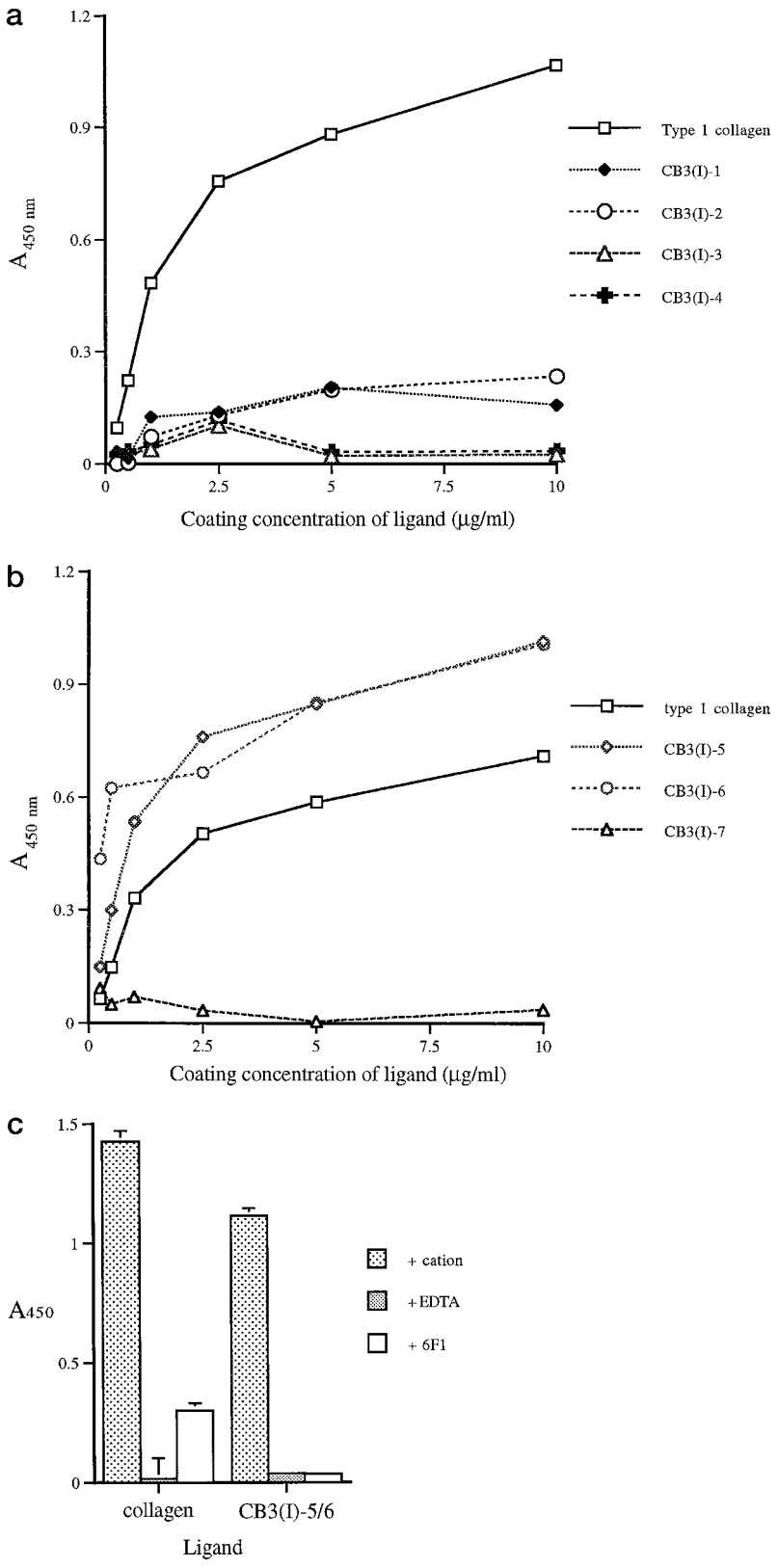

FIG. 3. **$\alpha_2\beta_1$ binding to immobilized collagen and α_1(I)CB3-derived peptides.** *a*, binding to collagen and peptides CB3(I)-1 to -4. *b*, binding to collagen and peptides CB3(I)-5 to -7. Points are the mean of triplicate determinations. Results are representative of three repeat experiments. *c*, binding to collagen and CB3(I)-5/6, for each of which the coating concentration was 10 μg/ml. *Cation* refers to binding in presence of 2 mM Mg^{2+}. The effect of 10 mM EDTA and preincubation with 2 μg/ml mAb 6F1 is shown. Data are the mean of triplicate determinations ± S.D. Error bars are absent when too close to reproduce. Data are representative of two repeat experiments.

collagen-related peptide GCP*(GPP*)$_{10}$GCP*G is largely cation-independent, the small amount of cation-dependent adhesion being secondary to platelet activation (15). In the present study, all the CB3(I)-derived peptides readily supported platelet adhesion, as was the case in our previous study of peptides based on the collagen III fragment α_1(III)CB4 (18). In all cases, adhesion was partly divalent cation-dependent. However, only in the case of peptides CB3(I)-5, -6, and -5/6 was this cation-dependent element of adhesion susceptible to blockade with mAb 6F1 (Table I).

Platelet Aggregation by CB3(I)-1 to -7 and CB3(I)-5/6—Following cross-linking, peptides CB3(I)-1 to -6 and CB3(I)-5/6 all showed substantial platelet aggregatory activity when tested at 20 °C. Peptides CB3(I)-6 and -5/6 were consistently more

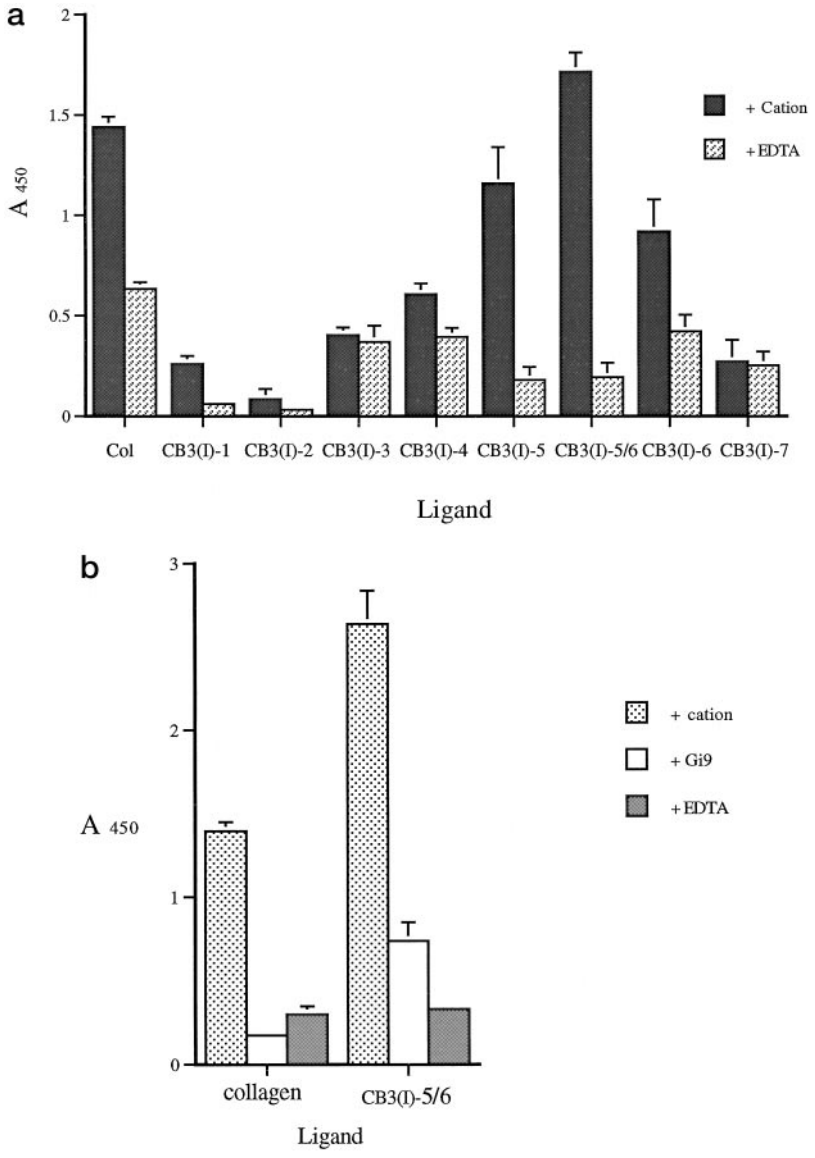

FIG. 4. **α_2 A-domain binding to immobilized collagen and α_1(I)CB3-based peptides.** Substrate in all cases was coated at 10 μg/ml. Where indicated, EDTA (10 mM) or Mg^{2+} (2 mM) was present. *a*, binding to collagen and peptides CB3(I)-1 to CB3(I)-7 and peptide CB3(I)-5/6. *b*, binding to collagen and peptide CB3(I)-5/6, and after preincubation with the anti-α_2 mAb Gi9 (10 μg/ml) as indicated. Results are the mean of triplicate determinations ± S.D. and are representative of two replicate sets of experiments. Where error bars are missing, they were too close to show.

active than collagen fibers. Unexpectedly, CB3(I)-7 was without activity, even when tested at up to 2 mg/ml. The minimum concentration for activity was as follows: CB3(I)-1 to -3, 1 μg/ml; CB3(I)-4, 5 μg/ml; CB3(I)-5, 10 μg/ml; CB3(I)-6 and -5/6, 0.1 μg/ml; CB3(I)-7, > 2.0 mg/ml. For comparison, fibers were active at 0.5 μg/ml. Aggregation stimulated by CB3(I)-5/6 and the inactivity of CB3(I)-7 are shown in Fig. 7.

DISCUSSION

The $\alpha_2\beta_1$-binding Sequence—In this study we have identified in the bovine collagen type I fragment α_1(I)CB3 a 15-residue sequence, GFP*GERGVEGPP*GPA, corresponding to residues 502–516 of the parent α_1(I) chain, that, on the basis of $\alpha_2\beta_1$ and α_2 A-domain binding and $\alpha_2\beta_1$-mediated cell adhesion, is an $\alpha_2\beta_1$ recognition sequence. From studies of α_2 A-domain binding, we have concluded that the GER triplet is essential for activity, while the Glu in the GVE triplet appears to be unimportant. A GER triplet at this locus is also present in the bovine α_2(I) chain and in human α_1(I) and α_2(I) chains (30). In an earlier study (18), we identified part of an $\alpha_2\beta_1$ recognition site in the bovine collagen α_1(III) chain, residues 522–528. We speculated that the GER sequence close by, equivalent to the GER triplet in the α_1(I) sequence studied here, may be involved in $\alpha_2\beta_1$ recognition. Evidence presented here for the involvement of a Glu residue in the recognition of $\alpha_2\beta_1$ is particularly intriguing in the light of the crystal structure of the α_2 A-domain where modeling studies indicated that a Glu (rather than an Asp) in a collagen triple helix could coordinate with a Mg^{2+} bound to the integrin A-domain metal ion binding site (31).

The GER motif within the $\alpha_2\beta_1$-binding sequence shows distinct resemblance to the $\alpha_1\beta_1$ recognition site in collagen IV which involves an Asp (rather than Glu) residue at position 461 in the α_1(IV) chain and an Arg residue at the same residue position (461) in the α_2(IV) chain (32). In our case, the crucial Glu and Arg residues occur in adjacent positions in the same α chain. We do not know as yet whether recognition involves the Glu and Arg residues in the same or in adjacent chains of the triple-helical structure. It is also clear that not all GER sequences in collagen can be acting as $\alpha_2\beta_1$ recognition sites since, for example, a GER sequence is present in the inactive peptides CB3(I)-2 and -3. It is of interest that CB3(I)-1, which also contains a GER triplet, can support $\alpha_2\beta_1$-mediated adhesion of HT 1080 cells. This may suggest that $\alpha_2\beta_1$ expressed by HT 1080 cells is slightly different to platelet $\alpha_2\beta_1$. It is known, for example, that $\alpha_2\beta_1$ exhibits a different ligand specificity when expressed on other cell types (33, 34).

Binding of the α_2 A-domain to peptide CB3(I)-5/6 was con-

sistently greater than to collagen. The reason for this is not clear, but may reflect a higher density of binding sites in the immobilized peptide than presented by immobilized collagen.

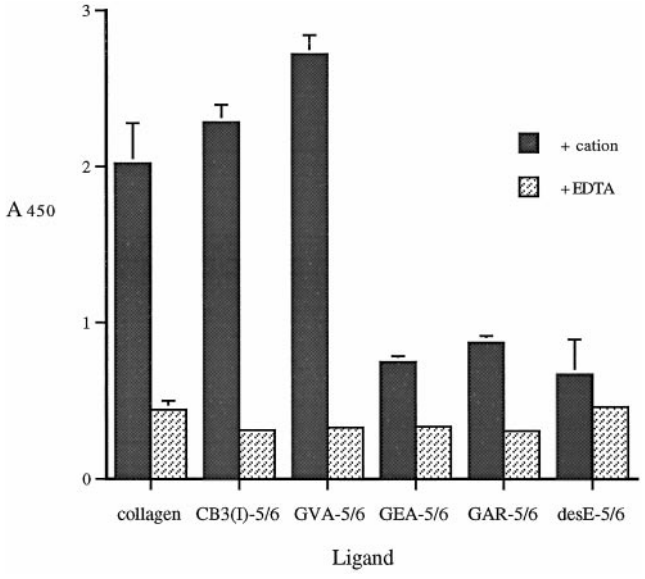

FIG. 5. **Binding of α_2 A-domain to peptide CB3(I)-5/6 and its variants.** Binding was measured in the presence of 2 mM Mg^{2+} or 10 mM EDTA. Substrate in all cases was coated at 10 μg/ml. Results are the mean of three determinations ± S.D. Data are representative of two repeat experiments.

Conversely, $\alpha_2\beta_1$ binding was greater to collagen than to CB3(I)-5/6, perhaps reflecting enhancement of collagen binding to $\alpha_2\beta_1$ A-domain by site(s) in the adjacent EF hand domain (35).

TABLE I

Effect of the anti-(α_2 subunit) mAb 6F1 on the divalent cation-dependent platelet adhesion to α_1CB3-based peptides

Adhesion was measured in 96-well plates at 20 °C using ^{51}Cr-labeled gel-filtered platelets and is expressed as the percentage of total radioactivity bound. Total adhesion of platelets was measured in the presence of 2 mM Mg^{2+} and cation-independent adhesion in the presence of 2 mM EDTA. Collagen and peptides were coated at 10 μg/ml. The anti-α_2 mab, 6F1, was tested at 2 μg/ml, and its effect is expressed as percentage inhibition of the cation-dependent adhesion. Values are the mean of triplicate determinations, and errors did not exceed 10% of the mean.

Substrate	Adhesion (% of total radioactivity bound)				Inhibition by 6F1 of cation-dependent adhesion
	Total (+Mg^{2+})	$\alpha_2\beta_1$-independent (+6F1)	Cation-independent (+EDTA)	Cation-dependent (col 2–col 4)	
					%
Collagen	15	1	1	14	100
CB3(I)-1	15	16	10	5	0
CB3(I)-2	16	17	8	8	0
CB3(I)-3	13	15	9	4	0
CB3(I)-4	14	14	5	9	0
CB3(I)-5	17	7	2	15	65
CB3(I)-6	14	7	5	9	80
CB3(I)-7	12	13	8	4	0
CB3(I)-5/6	21	16	15	6	85

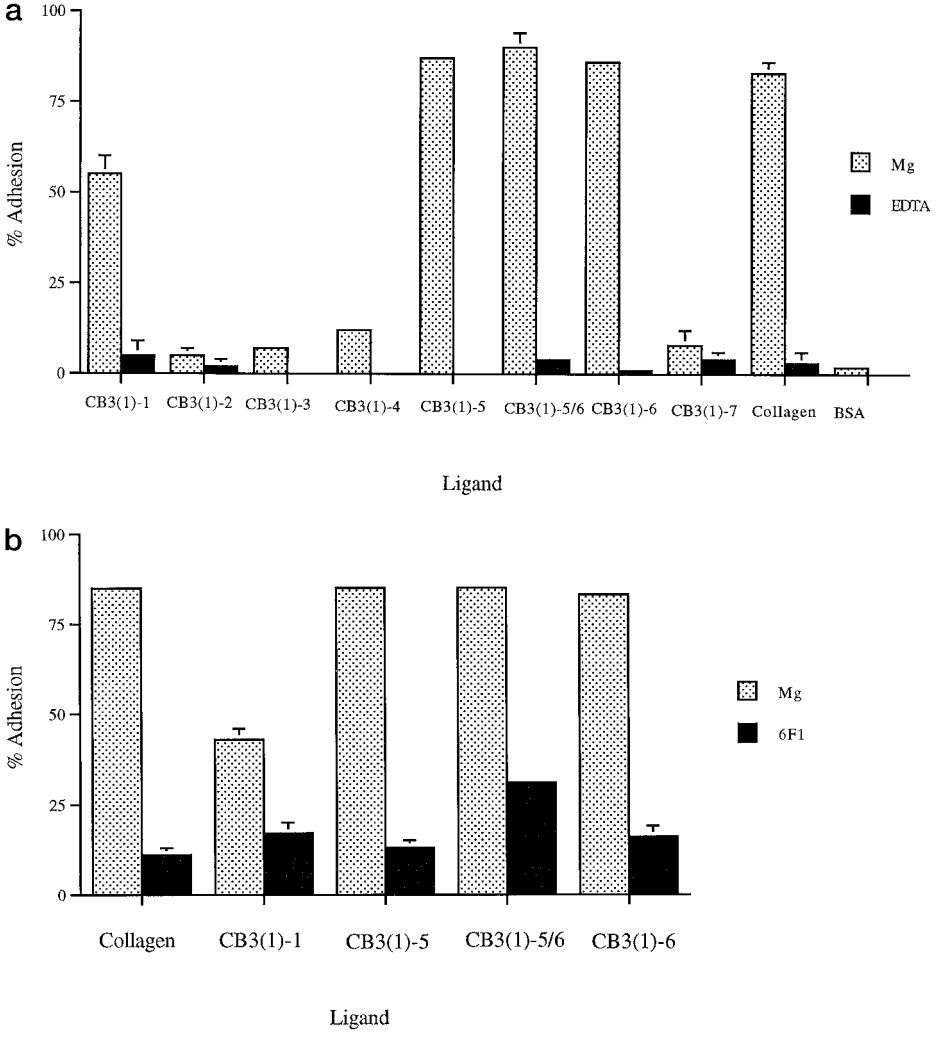

FIG. 6. **HT 1080 cell adhesion to collagen and peptides CB3(I)-1 to -7 and CB3(I)-5/6.** Adhesion, expressed as the number of adherent cells as a percentage of the total cell count, was measured at 20 °C in 96-well plates at 90 min. Wells were coated with collagen or peptide at 10 μg/ml. Results are the mean of triplicate determinations ± S.D. Where error bars are not shown, they were too close to reproduce. Data are representative of three repeat experiments. *a*, divalent cation-dependent adhesion. Adhesion was measured in the presence of 2 mM Mg^{2+} or 10 mM EDTA as indicated. *b*, effect of anti-α_2 mAb 6F1. Adhesion was measured in the presence or absence of 6F1 (2 μg/ml) as indicated. Data have been corrected for background adhesion using BSA.

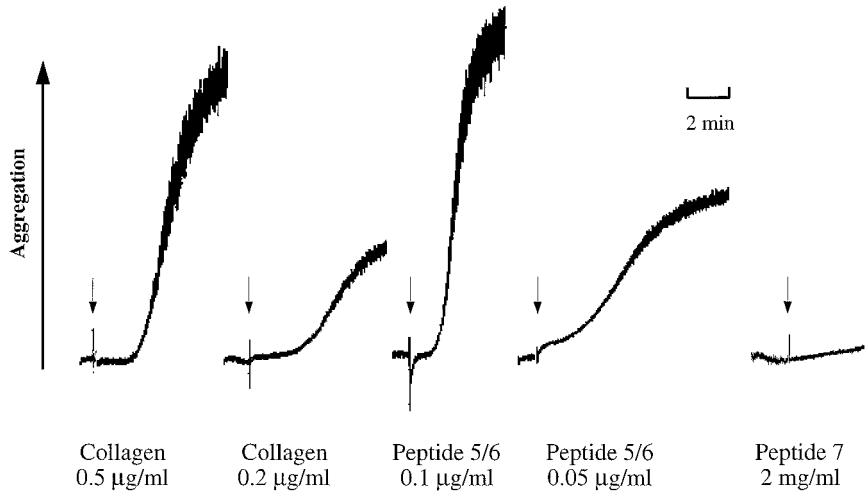

FIG. 7. **Platelet aggregometry in the presence of collagen, peptide CB3(I)-5/6, and the inactive peptide CB3(I)-7.** The *arrows* indicate the point of addition of ligand at the final concentration specified. Aggregation at 20 °C is shown as an increase in light transmittance.

HT 1080 cell adhesion to collagen is mediated predominantly by a single $\alpha_2\beta_1$-dependent mechanism. In contrast, platelet adhesion to collagen is a complex phenomenon; while adhesion to immobilized monomeric collagen is a divalent cation-dependent process mediated by $\alpha_2\beta_1$ (4, 9, 10, 14, 16, 29), adhesion to collagen fibers reveals a cation-independent element (9) involving other receptors such as Gp VI and CD 36 (29). All the peptides studied here revealed both cation-dependent and -independent adhesion. We assumed any effect of 6F1 to be directed against cation-dependent adhesion, since it is well established that divalent cations (Mg^{2+}) are essential for $\alpha_2\beta_1$ function.

As regards platelet adhesion, only the three peptides CB3(I)-5, -6, and -5/6 revealed any sensitivity to 6F1, confirming the location of an $\alpha_2\beta_1$ recognition site in the 5/6 overlap sequence. Cation-dependent adhesion to peptides which is unresponsive to 6F1 probably involves a secondary process of attachment subsequent to primary adhesion which might be mediated by Gp VI. We have observed a similar phenomenon using peptides consisting solely of repeat GPP* sequences where primary cation-independent adhesion (mediated by Gp VI)[3] is enhanced by a secondary cation-dependent process (15). In other studies,[3] we have found that a peptide consisting of the CB3(I)-5/6 overlap sequence inserted within repeat GPP (rather than GPP*) triplets, fails to exhibit any cation-independent adhesion, but rather, like monomeric collagen, supports cation-dependent adhesion fully mediated by $\alpha_2\beta_1$. This observation supports the view that GPP* sequences in the peptides are responsible for cation-independent adhesion (via Gp VI) and confirms the identification of the 5/6 overlap sequence as an $\alpha_2\beta_1$ recognition sequence. The higher cation-independent adhesion to CB3(I)-5/6 relative to CB3(I)-5 and -6 probably reflects its higher relative content of GPP* triplets.

From inhibition studies using short, linear (non-helical) peptides, Santoro and colleagues (11) identified the sequence DGEA, corresponding to residues 435–438 of the α_1(I) chain, as an $\alpha_2\beta_1$ recognition site. This sequence occurs in CB3(I)-2 of the present study. However, this peptide failed to bind $\alpha_2\beta_1$ or the α_2 A-domain or to support $\alpha_2\beta_1$-mediated adhesion. Fields and colleagues (36) have also reported that a triple-helical peptide containing DGEA was unable to support integrin (β_1)-mediated fibroblast adhesion.

Platelet-aggregatory Activity of CB3(I)-1 to -7—We proposed previously that the platelet-aggregatory activity of collagen might be an intrinsic property of the triple helix. This activity could be modified by the presence of sequences that might enhance activity, for example $\alpha_2\beta_1$ recognition sequences, or might diminish activity, for example, by exhibiting a preponderance of negative charges (7, 15, 18). Our current studies indicate that the basic aggregatory activity of collagen is not simply a recognition of the triple helix *per se* but rather a highly specific recognition by platelet Gp VI of GPP* sequences within the collagen triple helix.[3] The aggregatory activity of CB3(I)-1 to -6 may be due in large part to the inclusion of the terminal GPP* sequences necessary to ensure the triple-helical conformation required for platelet reactivity (28). Nevertheless, although all the peptides have the same $(GPP^*)_3$ sequence at either end, they reveal a considerable range of activity with the most active able to aggregate platelets at 0.1 μg/ml, whereas others are only active at 10 μg/ml and above. The high activity of peptides CB3(I)-6 and -5/6 might be attributable to the presence of the $\alpha_2\beta_1$ recognition sequence. However, against this, peptide CB3(I)-5, also containing this sequence, is of relatively poor activity. Remarkably, CB3(I)-7, despite having the $(GPP^*)_3$ repeat at either end, was unable to induce platelet aggregation. This might indicate the presence in CB3(I)-7 of an inhibitory sequence that could possibly account for the inactivity of the parent fragment α_1(I)CB3. However, we cannot exclude the possibility that the inactivity of the fragment might be due to an inappropriate distribution or inadequate density of GPP* triplets. Interestingly, the equivalent collagen III fragment, α_1(III)CB4, which possesses good aggregatory activity, contains twice as many GPP* triplets as α_1(I)CB3 (18). As speculated previously (18), the relative lack of activity of α_1(I)CB3 might also be attributable in part to an excess of negative charges.

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PROTEIN CHEMISTRY AND STRUCTURE:

Identification in Collagen Type I of an Integrin $\alpha_2\beta_1$-binding Site Containing an Essential GER Sequence

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