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 $a_1(I)CB3$ 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 $a_2\beta_1$ and the recombinant $a_2$ A-domain, and their ability to support $a_2\beta_1$-mediated cell adhesion, we identified two peptides, CB3(I)-5 and -6, which contain an $a_2\beta_1$ 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 $a_1$ 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 platelet-activatory ability, we have synthesized the fragment as seven overlapping peptides that spontaneously assemble into triple helices. Based on their ability to support integrin-mediated adhesion, it largely lacks the capacity to activate platelets (12, 13), indicating that recognition of $a_2\beta_1$ 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 $a_1(I)CB3$ recognizing $a_2\beta_1$, 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 $a_2\beta_1$-mediated adhesion of platelets and HT 1080 cells and to bind the purified integrin and the recombinant $a_2$ A-domain, we have identified a 15-residue $a_2\beta_1$ recognition sequence, corresponding to residues 502–516 of the $a_1$ chain, containing a GER triplet crucial for activity. We were unable to detect activity within the peptide CB3(I)-2 containing the sequence DGEA, corresponding to residues 435–438 of the $a_1$ chain, is an $a_2\beta_1$ recognition sequence in $a_1(I)CB3$ (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 $a_2\beta_1$ 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 $a_1(I)CB3$ recognizing $a_2\beta_1$, 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 the presence of an inhibitory element that might account for the lack of aggregatory activity of the parent $a_1(I)CB3$ 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 $a_2\beta_1$ 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 Ia/IIa 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 $a_1(I)CB3$ derived from the $a_1$ 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 $a_1$ chain, is an $\alpha_2\beta_1$ recognition sequence in $a_1(I)CB3$ (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 $a_1(I)CB3$ 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 $\alpha_2$ A-domain, we have identified a 15-residue $\alpha_2\beta_1$ recognition sequence, corresponding to residues 502–516 of the $a_1$ chain, containing a GER triplet crucial for activity. We were unable to detect activity within the peptide CB3(I)-2 containing the sequence DGEA, corresponding to residues 435–438 of the $a_1$ chain, is an $\alpha_2\beta_1$ recognition sequence in $a_1(I)CB3$ (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 $a_1(I)CB3$ 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 $\alpha_2$ A-domain, we have identified a 15-residue $\alpha_2\beta_1$ recognition sequence, corresponding to residues 502–516 of the $a_1$ 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, diazoylated 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 α2b-integrin subunit mAb 6F1 (14) was a generous gift from Dr. B. S. Coller (Mount Sinai Hospital, New York, NY). Anti-human integrin α2b-1 mAb, clone 6G9, was purchased from The Binding Site Ltd. (Birmingham, United Kingdom (UK)).

Fmoc-amino acids were from Alexis Corp. (Nottingham, UK). Fmoc-Asp(tert-butyli ester)-pentafluorophenyl ester and Fmoc-(Fmoc-2-hydroxy-4-methylbenzyl)Gly-ÖH were from Calbiochem-Novabiochem (UK Ltd.). (Nottingham, UK). N-{Diallylamino}-1H-1,2,3-triazol-5-1,5-bipyrind-4-imethylenemethanamnium hexafluorophosphosphate 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 3Cr-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 concanavalin A never more than 1%.

When testing mAbs for inhibitory activity, platelets were pre-incubated with 100 μg/ml of polyclonal rabbit anti-glutathione S-transferase in TBS containing 1 mM MgCl2 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 Mg2+ 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-Synthesizer. In general, Fmoc-amino acids (4 eq) were activated with N-{(diallylamino)-1H-1,2,3-triazol-4(5,6)-pyrind-1-yl-methylene}-N-methylmethanaminium hexafluorophosphosphate N-oxide (4 eq) in the presence of diisopropylethylamine (8 eq) (23). 7-Aza-1-hydroxybenzotriazole (4 eq) was added when coupling Aam 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 therefore, in the presence of 20 mM MgCl2 and 1 mg/ml BSA (Sigma A7638), 100 μl of 50 μM/ml of BSA, a domain-fusion protein in the above buffer or one containing CB3(1–5)-1,2,3-triazol-5(6)-pyridin-1-yl-methylene-N-methylmethanaminium hexafluorophosphosphate N-oxide and 7-aza-1-hydroxybenzotriazole was applied to the wells and the plates incubated for 3 h at room temperature. Wells were then washed as before. Bound mAb was detected by the addition of 100 μl of 10 μg/ml polyclonal rabbit anti-glutathione S-transferase in TBS containing 1 mM MgCl2 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 Mg2+ 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.

The assay procedure has been described in detail elsewhere (19, 20). Briefly, wells of 96-well enzyme-linked immunosorbent assay plates (Nunc Maxisorb) 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 MgCl2 and 1 mg/ml BSA (Sigma A7638), 100 μl of a solution of 50 μg/ml BSA, a domain-fusion protein in the above buffer or one containing CB3(1–5)-1,2,3-triazol-5(6)-pyridin-1-yl-methylene-N-methylmethanaminium hexafluorophosphosphate N-oxide and 7-aza-1-hydroxybenzotriazole was applied to the wells and the plates incubated for 2 h at 37 °C. Plates were again washed three times and bound integrin detected 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.

Integrin α2β1—Integrin α2β1 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 Integrin α2β1 was confirmed by reverse phase high performance liquid chromatography on a column of Vydac 219TP1015/22 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 CB3(1–5), designated CB3(1–1) to 7, were synthesized (see Fig. 1). Subsequently, a peptide designated CB3(1–5)/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 GPPα 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 α2β1-mediated cell adhesion (9, 18, 28). The GPPα 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 (Tm) of the peptides were as follows: CB3(1–1), 32 °C; CB3(1–2), 39 °C; CB3(1–3), 37 °C; CB3(1–4), 36 °C; CB3(1–5), 30 °C; CB3(1–6), 30 °C; CB3(1–7), 26 °C; CB3(1–5/6),

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sequences corresponding to those in collagen (Fig. 3) 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 weaker binding to peptide CB3(I)-6, and is shown in bold. The sequences of the variants of CB3(I)-5/6 are also shown.

Fig. 1. Peptide sequences. For peptides CB3(I)-1 to -7, the sequences corresponding to those in α1(I)CB3 are shown in bold. The overlap between adjacent peptides is underlined. The seven peptides span the 149-residue length of α1(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 α2β1—In accord with previous findings (4, 19, 20), we could detect binding of α2β1 to collagen in solid-phase assays, which was largely divalent cation-dependent and strongly inhibited by anti-α2β1 mAbs (Fig. 3, a–c). Of the seven peptides CB3(I)-1 to -7, only peptides 5 and 6 showed α2β1 binding (Fig. 3, a and b). Binding to these two peptides was greater than to collagen (Fig. 4, a–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.
collagen-related peptide GCP\(^*(\text{GPP})^*\) 10GCP*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 \(\alpha_1(\text{III})\text{CB4}\) (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

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**FIG. 3.** \(\alpha_2\beta_1\) binding to immobilized collagen and \(\alpha_1(\text{I})\text{CB3}-\text{derived peptides.}\) 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 \(\mu\text{g/ml}\). Cation refers to binding in presence of 2 mM Mg\(^{2+}\). The effect of 10 mM EDTA and preincubation with 2 \(\mu\text{g/ml}\) mAb 6F1 is shown. Data are the mean of triplicate determinations \(\pm\) S.D. Error bars are absent when too close to reproduce. Data are representative of two repeat experiments.
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 mg/ml; CB3(I)-4, 5 mg/ml; CB3(I)-5, 10 mg/ml; CB3(I)-6 and -5/6, 0.1 mg/ml; CB3(I)-7, > 2.0 mg/ml. For comparison, fibers were active at 0.5 mg/ml. Aggregation stimulated by CB3(I)-5/6 and the inactivity of CB3(I)-7 are shown in Fig. 7.

**DISCUSSION**

The α₂β₁-binding Sequence—In this study we have identified in the bovine collagen type I fragment α₁(I)CB3 a 15-residue sequence, GFP*GERGVEGPP*GPA, corresponding to residues 502–516 of the parent α₁(I) chain, that, on the basis of α₂β₁ and α₂ A-domain binding and α₂β₁-mediated cell adhesion, is an α₂β₁ recognition sequence. From studies of α₂ 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 α₂(I) chain and in human α₁(I) and α₂(I) chains (30). In an earlier study (18), we identified part of an α₂β₁ recognition site in the bovine collagen α₁(III) chain, residues 522–528. We speculated that the GER sequence close by, equivalent to the GER triplet in the α₁(I) sequence studied here, may be involved in α₂β₁ recognition. Evidence presented here for the involvement of a Glu residue in the recognition of α₂β₁ is particularly intriguing in the light of the crystal structure of the α₂ A-domain where modeling studies indicated that a Glu (rather than an Asp) in a collagen triple helix could coordinate with a Mg²⁺ bound to the integrin A-domain metal ion binding site (31).

The GER motif within the α₂β₁-binding sequence shows distinct resemblance to the α₁β₁ recognition site in collagen IV which involves an Asp (rather than Glu) residue at position 461 in the α₁(IV) chain and an Arg residue at the same residue position (461) in the α₂(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 α₂β₁ 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 α₂β₁-mediated adhesion of HT 1080 cells. This may suggest that α₂β₁ expressed by HT 1080 cells is slightly different to platelet α₂β₁. It is known, for example, that α₂β₁ exhibits a different ligand specificity when expressed on other cell types (33, 34).

Binding of the α₂ A-domain to peptide CB3(I)-5/6 was con-
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Persistently 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.

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).

**Fig. 5.** Binding of $\alpha_2$ 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 $\mu$g/ml. Results are the mean of three determinations ± S.D. Data are representative of two repeat experiments.

**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 $\mu$g/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-$\alpha_2$ mAb 6F1. Adhesion was measured in the presence or absence of 6F1 (2 $\mu$g/ml) as indicated. Data have been corrected for background adhesion using BSA.

**Table I**

| 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) | Cation-dependent (col 2–col 4) |
| Collagen  | 15 1 1 14 100 | 0 | 0 | 0 | 0 |
| CB3(I)-1  | 15 16 10 5 0 | 0 | 0 | 0 | 0 |
| CB3(I)-2  | 16 17 8 8 0 | 0 | 0 | 0 | 0 |
| CB3(I)-3  | 13 15 9 4 0 | 0 | 0 | 0 | 0 |
| CB3(I)-4  | 14 14 5 9 0 | 0 | 0 | 0 | 0 |
| CB3(I)-5  | 17 7 2 15 65 | 80 | 80 | 80 | 80 |
| CB3(I)-6  | 14 7 5 9 80 | 80 | 80 | 80 | 80 |
| CB3(I)-7  | 12 13 8 4 0 | 0 | 0 | 0 | 0 |
| CB3(I)-5/6| 21 16 15 6 85 | 85 | 85 | 85 | 85 |
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 component (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 ($\text{Mg}^{2+}$) 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) is enhanced by a secondary cation-dependent process (15).

In other studies, 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 $\alpha_1$-I chain, as $\alpha_2\beta_1$-mediated fibroblast adhesion. Peptides containing DGEA was unable to support integrin ($\alpha_2\beta_1$)-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. 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 $\mu$g/ml, whereas others are only active at 10 $\mu$g/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 $\alpha_1$(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, $\alpha_1$(III)CB4, which possesses good aggregatory activity, contains twice as many GPP* triplets as $\alpha_1$(I)CB3 (18). As speculated previously (18), the relative lack of activity of $\alpha_1$(I)CB3 might also be attributable in part to an excess of negative charges.

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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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