Streptococcus sanguis expresses a cell wall-bound protein that induces the activation and aggregation of platelets. This platelet aggregation-associated protein (PAAP) contains a collagen-like, platelet-interactive domain within a 23-kDa protein fragment. To isolate the minimal platelet-interactive domain, p23 PAAP was digested with collagenase, and the digest chromatographed to isolate fractions with activity inhibitory to S. sanguis-induced platelet aggregation. The active fraction was then digested with cyanogen bromide, the product chromatographed, and a smaller inhibitory peptide isolated. Finally, this fraction was digested with endoproteinase Lys-C, and the digest fractionated. After each step, inhibitory activity resolved into a single copy of the platelet-interactive domain. The minimal 7-mer peptide was purified by immunoaffinity chromatography and reverse phase high pressure liquid chromatography. The primary structure was determined to be Pro-Gly-Glu-Gln-Gly-Pro-Lys. This sequence conforms to the predicted structural motif of the platelet-interactive domains of types I and III collagen. Its structure explains the molecular basis for immunological cross-reactivity and functional similarity to the platelet-interactive domains of collagens.

Certain strains of Streptococcus sanguis express surface fibrils that induce human platelets in plasma to aggregate in vitro (1). In vivo, proteins from these fibrils are directly involved in the formation of the thrombi and the vegetations of experimental endocarditis (2). Aggregation of platelets in response to cells of S. sanguis is mediated by at least two different classes of antigens on the surface fibrils (1, 3). The class I antigen(s) mediates adhesion or direct binding to platelets. The class II antigen then interacts with its specific receptors, triggering the up-regulation of platelet glycoprotein Ib-IIIa, the fibrinogen receptor. Cross-linked by fibrinogen, the platelets then aggregate.

The class II, or platelet-aggregation-associated protein (PAAP), has a functional domain located in a 23-kDa segment of a 65-kDa cell wall-bound protein fragment (4) and in exported and released precursor glycoproteins of 111 and 115 kDa, respectively (5). The PAAP is immunologically cross-reactive with types I and III collagens, which also induce platelets to aggregate. Monospecific antibodies against the PAAP react with types I and III collagen to inhibit induction of platelet aggregation (6). Antibodies against types I, III, and IV collagens also react with cell-associated PAAP to inhibit S. sanguis-induced platelet aggregation (7). These anti-PAAP and anti-collagen antibodies are specifically neutralized by incubation with soluble PAAP or PAAP fragments or with a synthetic peptide, KPGEPGPK, patterned from platelet-interactive domains on collagens (6–8). To understand further the molecular basis of streptococcal-platelet interactions, we sought to purify and characterize the minimal functional domain of this collagen-like PAAP from S. sanguis. This report describes the isolation and purification of a heptapeptide fragment, KPGEPGPK, which retains platelet-interactive activity.

**EXPERIMENTAL PROCEDURES**

**Microorganisms: Growth and Preparation**—The prototypic Adh+Agg+ strain, S. sanguis 1, is grown overnight in Todd-Hewitt broth in 5% CO2, harvested by centrifugation (7,500 × g, 20 min, 4°C), and washed twice in cold 0.01 M sodium phosphate buffer, pH 7.0, with 0.9% (w/v) sodium chloride (phosphate-buffered saline). After washing, the bacteria were dispersed and suspended to 4 × 108 cells/ml in phosphate-buffered saline precisely as described in previous reports (1, 3, 9).

Whole cells of S. sanguis required for protoplast conversion were initially grown in the liquid chemically defined synthetic medium (FMC) (11), harvested, washed, and treated with mutanolysin as described previously (5). Protoplasts were cultured in fresh FMC in the presence of penicillin. Every 24 h the spent medium was collected, from which PAAP was isolated, and fresh FMC was added to the continuing culture of protoplasts.

**Preparation of Anti-KPGEPGPK Immunoadfinity Matrix**—Female New Zealand White rabbits were immunized to prepare monospecific antisera to KPGEPGPK conjugated to bovine serum albumin as previously described (7). γ-Globulin was isolated from whole serum by precipitation with saturated ammonium sulfate (12). The IgG was purified sequentially by ion exchange and gel filtration column chromatography (4). Purified anti-KPGEPGPK IgG (5 mg) was coupled to 10 ml of cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) according to the manufacturer’s protocol. The coupled gel was packed into a column (1.6 × 9 cm), and 1 The abbreviations used are: PAAP, platelet aggregation-associated protein; HPLC, high pressure liquid chromatography; PRP, platelet-rich plasma.
washed, and equilibrated with 20 volumes of 0.1 M Tris, pH 8.5.

**Proteolytic Cleavage of Isolated PAAP—**Protocols for the purification of the PAAP as a 115-kDa glycoprotein from proteolipid culture medium (5) or a 65-kDa cell wall protein fragment (4) have been described earlier. The 23-kDa platelet-interactive fragment was purified from the cell wall preparation as described previously (4). Isolated p23 PAAP (8 mg) was reduced and S-carboxymethylated (13), and digested with bacterial collagenase type IA (Sigma; 50 mM Tris, pH 7.4, with 40 mM NaCl and 5 mM CaCl₂, 24 h, 24 °C; E/P ratio, 1:15). Digestion was stopped by the addition of acetic acid to reduce the pH to 4.2. The resulting fragments were concentrated and desalted by ultrafiltration (VYOS membrane, 500 MWCO, Amicon Corp.), fractionated on a column of Sephadex G-50 (0.8 cm × 50 cm, 20 ml/min, HzO, 24 °C), and tested for the ability to inhibit S. sanguis-induced platelet aggregation. The platelet-interactive protein fragment, p13 PAAP (2 mg), was then cleaved with cyanogen bromide (CNBrprotein weight ratio of 2:1; formic acid, 4 h, room temperature). The digestion was stopped by 10-fold dilution with distilled HzO and immediate lyophilization. The resulting fragments were fractionated by HPLC using a DEAE-PiW column (Waters Associates, Milford, MA; 50 mM Tris, pH 8.0, with 20–500 mM NaCl, 1 ml/min), concentrated and desalted by ultrafiltration, and tested for platelet activity. The active protein fragment, p2.7 PAAP (0.3 mg), was digested with endoproteinase Lys-C (25 mM Tris-HCl, pH 8.5, with 1 mM EDTA, 24 °C, 18 h; E/P ratio, 1:50), lyophilized to stop digestion, and fractionated sequentially by: (1) immunoaffinity chromatography on an anti-KPGEPGPK column (washed with 0.1 M Tris, pH 8.5, to remove unbound materials; bound materials were eluted with 0.1 M Tris, pH 2.5); and (2) reverse phase HPLC (Zorbax Protein Plus column, MacMod Analytical, Chadds Ford, PA; 0.1% trifluoroacetic acid in a gradient of 5–70% acetonitrile, 2 ml/min). Fractions were pooled by absorbance at 214 nm and lyophilized.

**Platelet Aggregometry—**S. sanguis was tested for the ability to induce platelet aggregation with fresh platelet-rich plasma (PRP) obtained from a single donor by a method described in earlier reports (3, 9, 14, 15). In some experiments, 0.4 ml of PRP containing about 2 × 10⁸ platelets was preincubated for 10 min at 37 °C in stirred cuvettes with 0.05 ml of purified PAAP or PAAP protein fragments. In all cases, 0.05 ml of streptococcal suspension containing 4 × 10⁶ cells/ml were then incubated with each PRP suspension. PRP aggregation was performed at 37 °C with controlled stirring in a recording aggregometer (model 390, Chronolog Corp., Broome, PA), and the lag time or delay to onset (minutes) was measured.

**Amino Acid Analyses—**Amino acid compositions were determined by the high pressure liquid chromatography procedures of Bidlingmeyer et al. (16) as previously described (4). Amino acid sequence analyses were performed by the Microchemical Facility of the University of Minnesota by automated Edman degradation using an Applied Biosystems Sequencer with on-line, reverse-phase chromatographic analysis of the phenylthiohydantoin derivatives.

**Electrophoretic Analyses—**SDS-polyacrylamide gel electrophoresis was performed as previously described (17). Samples were incubated in 1% (w/v) SDS sample buffer with or without 2-mercaptoethanol for 2 min in a boiling water bath before application to the gel. After electrophoresis, acrylamide gels were washed overnight in 50% (v/v) methanol (18) and stained with methylarmochromatic silver (19).

**RESULTS**

To obtain a smaller biologically active fragment, p23 PAAP was reduced, carboxymethylated, and digested with collagenase. The resulting fragments (92% recovery) were fractionated on a column of Sephadex G-50 (Fig. 1). Platelet-interactive activity was confined to a single 13.6-kilodalton fragment. Amino acid analysis revealed the presence of 3 residues of methionine (Table I). This p13 PAAP fragment was then digested with cyanogen bromide. The resulting fragments (81% recovery) were fractionated by HPLC ion exchange chromatography (Fig. 2A). Rechromatography in the same conditions (Fig. 2B) demonstrated the relative purity of the single peak that contained biologic activity. Amino acid analysis showed that the active peptide contained 28 amino acids with a minimum residue weight of 2766 daltons and was enriched in Gly, Lys, and Pro (Table I). This p2.7 PAAP peptide fragment contained 24 times the activity, on a weight basis, when compared with the parent p115 PAAP (Table II).

The p2.7 PAAP peptide fragment was then digested with endoproteinase Lys-C. The resulting fragments were placed on an anti-KPGEPGPK immunofluorinity column. After washing the column with equilibrating buffer, the bound peptide was eluted under mild acid conditions. This peptide was further purified by reverse phase HPLC chromatography. The amino acid composition was determined (Table I). Automated N-terminal sequence analysis showed that the minimal platelet-interactive peptide fragment had the sequence, PGENGPK, which matched the consensus sequence reported for platelet-interactive domains of human and bovine types I and III collagens (7). This consensus sequence is also found in collagen types other than I and III from various species, including humans (Table III).

**DISCUSSION**

Viridans streptococci are predominant members of the commensal oral flora (20). Considered to be of low virulence...
been shown to be present on the cell surface of certain strains of inoculated cells of *S. sanguis*, from dose-response inhibition experiments. The mechanism employed by *S. sanguis* is under investigation by use of inoculated cells of *S. sanguis*.

In vitro, the components needed to promote adhesion to platelets and to induce subsequent platelet aggregation have been shown to be present on the cell surface of certain strains of *S. sanguis*. One of these components, PAAP, contains a determinant that is functionally and immunologically cross-reactive with the platelet-interactive domains on types I and III collagens (6, 7). These interstitial collagens are exposed to platelets following blood vessel injury. At the site of injury, these collagens fulfill an essential protective function, causing the aggregation of blood platelets and the formation of a hemostatic plug responsible for the arrest of bleeding. Human platelets that are specifically defective in response to collagen also fail to aggregate after challenge with cells of *S. sanguis* (15). Antibodies against the *S. sanguis* PAAP or against types I and III collagen react with cells of *S. sanguis* to inhibit their induction of platelet aggregation. These antibodies are specifically neutralized by soluble PAAP or PAAP fragments or synthetic peptide, Lys-Pro-Gly-Glu-Pro-Gly-Pro-Lys, which is an analogue of the platelet-interactive domain on cyanogen bromide fragment 4 (CB4) of type III collagen (8). Recently, a panel of synthetic peptides containing specific amino acid substitutions within this collagen-derived octapeptide sequence was used to predict a structural motif of the cross-reactive platelet-interactive domains (7). The predicted structural motif consisted of a negative charge surrounded by amino acids with high β-turn potential (7). This motif is characterized by the consensus sequence X-Pro-Gly-Glu-Pro/I/ and protein fragments and peptides with the consensus sequence XPGEP/QGPK, which other inhibitors block the binding of platelets to immobilized collagen substrates. Therefore, these other proteins and peptides may simulate different domains within collagen that participate in adhesion with platelets.

Recently, other peptides from collagen (22, 23) and a salivary protein from the *Haemoprya officinalis* leech (24, 25) have been implicated as inhibitors of collagen-induced platelet activation and aggregation. Unlike the collagen-derived octapeptide, KPGEPGPK, and protein fragments and peptides with the consensus sequence XPGE/#QGX, these other inhibitors block the binding of platelets to immobilized collagen substrates. Therefore, these other proteins and peptides may simulate different domains within collagen that participate in adhesion with platelets.

We have recently proposed that the platelet binding and the aggregation-inducing sites within the collagen molecule are distinct (7). Indeed, from this and other studies a model for collagen-platelet interactions has emerged. Adhesion to human platelets involves a specific domain or set of domains on the collagen molecule. The tetrapeptide, DGEA, described by Staatz et al. (22) may represent one such domain. Platelet access to another polymeric domain or set of domains is required to induce the aggregation response.

Recent research has focused on the identification of matching sequences in other collagen types which fit the platelet-interactive structural motif. Table III lists these sequences along with the source species, residues, sequence, and reference.

### Table III

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>Residues*</th>
<th>Sequence†</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2(IV) collagen</td>
<td>Human</td>
<td>403-409</td>
<td>PGEmGPK</td>
<td>26</td>
</tr>
<tr>
<td>Mouse</td>
<td>401-407</td>
<td>PGEmGPK</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>279-286</td>
<td>PGEmGPK</td>
<td>28</td>
<td></td>
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<tr>
<td>α1(VIII) collagen</td>
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<td>292-298</td>
<td>PGEmGPK</td>
<td>29</td>
</tr>
<tr>
<td>α2(IX) corneal collagen</td>
<td>Chicken</td>
<td>27-33</td>
<td>PGEmGPK</td>
<td>30</td>
</tr>
<tr>
<td>α1(XI) collagen</td>
<td>Human</td>
<td>120-126</td>
<td>PGEmGPK</td>
<td>31</td>
</tr>
</tbody>
</table>

* Residue numbers identifying sequences which fit the predicted structural requirements of two regions of β-turn potential surrounding an acidic amino acid within a 6-8-residue portion of the polypeptide.

† Capital letters indicate residues which are identical to the platelet-interactive consensus sequence of PGEP/QGPK.
(23) have shown that by chemical modification of amino acid side chains within collagen, platelet adherence was altered independently of activation and aggregation. Available aminogroups of collagen were suggested to be essential for the activation and aggregation of platelets. The platelet-interactive consensus sequence we have identified is frequently flanked by lysyl residues, such as in the collagen-derived octapeptide. While flanking lysine residues may not be required by platelets to recognize the consensus sequence, their modification can substantially alter the platelet interactivity of this domain (7). Therefore, they may stabilize binding. It is noteworthy that, like collagen, cells of S. sanguis express a separate adhesive determinant that is antigenically distinct from the PAAP (1). The PAAP antigen on cells of S. sanguis and human and animal collagens contain, therefore, domains that contribute selectively to the activation and aggregation of platelets with the structural motif provided by the expression of the consensus sequence XPGEP/QGPX. This domain in the soluable, cell-free form acts to inhibit the induction of platelet aggregation in response to either cells of S. sanguis or collagen. For this domain to induce aggregation of platelets depends upon the secondary and polymeric structure of the PAAP and collagen molecules. Polymeric, triple helical collagen is necessary to induce the aggregation of platelets, suggesting the need for multiple points of contact to produce a transmembrane signal (32–34). Barnes and co-workers (35) have demonstrated that glutaaldehyde-cross-linked polymers of several cyanogen bromide fragments from type I collagen were actually able to induce platelet aggregation. Their study demonstrated that collagen tertiary and quaternary structures are required for platelet aggregatory activity to be expressed. Recently, we have discovered that tryptic fragments of PAAP from cells of S. sanguis cultured in the presence of denatured collagen also were able to induce platelet aggregation. Since no hydroxyproline was detected in these peptides, it suggests that growth in collagen regulates the assembly and suprasecondary structure of PAAP on the fibrils of the cell surface.2

We have searched the GenBank data files to identify other domains which match our consensus sequence and structural motif using MELPROT (7). In addition to the platelet-interactive domains of bovine and human collagens previously reported (7), sequences from types IV, VIII, IX, and XI collagens were predicted to fit our criteria (Table III). This suggests that the platelet-interactive domain may be common in other collagens and participate in other interactions. Indeed, type IV collagen, containing a laminin-binding domain, was one of the identified matches. Recently (37), a collagen cross-reactive domain was demonstrated in a 145-kDa laminin-binding protein from Streptococcus gordonii strain 2316 (formerly classified as a strain of S. sanguis), which was also inducible by growth in collagen. While this protein has not been sequenced, it is likely that this S. gordonii protein may possess a domain analogous to the collagen-like domain we have identified in S. sanguis. Furthermore, this domain may be implicated in binding to laminin, thereby providing S. sanguis an initial strategy to adhere to injured, denuded heart valves.

Since autoantibodies against type II collagen are a common finding in patients with rheumatoid arthritis, we also analyzed the entire sequence of type II collagen with MELPROT. One domain was identified, which conformed to the structural motif and consensus sequence of the platelet-interactive domain (7). This domain, with the sequence Gly-Glu-Gln-Gly-Pro-Lys, is located at residues 142–147 of CB11. Interestingly, immunization of mice with this peptide has been demonstrated to suppress the development of arthritis (36). Therefore, the conformation and presentation of the platelet-interactive consensus sequence may be of fundamental importance in hemostasis, thrombosis, and, by virtue of its potential antigenicity (4, 6), rheumatoid arthritis.

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REFERENCES


P. R. Erickson and M. C. Herzberg, unpublished data.