The Tetrapeptide Analogue of the Cell Attachment Site of Fibronectin Inhibits Platelet Aggregation and Fibrinogen Binding to Activated Platelets*

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Fibrinogen binding to receptors on activated platelets is a prerequisite for platelet aggregation. However, the regions of fibrinogen interacting with these receptors have not been completely characterized. Fibronectin also binds to platelet fibrinogen receptors. Moreover, the amino acid sequence Arg-Gly-Asp-Ser, corresponding to the cell attachment site of fibronectin, is located near the carboxyl-terminal region of the α-chain of fibrinogen. We have examined the ability of this tetrapeptide to inhibit platelet aggregation and fibrinogen binding to activated platelets. Arg-Gly-Asp-Ser, but not the peptide Arg-Gly-Try-Ser-Leu-Gly, inhibited platelet aggregation stimulated by ADP, collagen, and γ-thrombin without inhibiting platelet shape change or secretion. At a concentration of 60-80 μM, Arg-Gly-Asp-Ser inhibited the aggregation of ADP-stimulated gel-filtered platelets ≥50%. Arg-Gly-Asp-Ser, but not Arg-Gly-Try-Ser-Leu-Gly, also inhibited fibrinogen binding to ADP-stimulated platelets. This inhibition was competitive with a K_i of ≥25 μM but was incomplete even at higher tetrapeptide concentrations, indicating that Arg-Gly-Asp-Ser is a partial competitive inhibitor of fibrinogen binding. These data suggest that a region near the carboxyl-terminus of the α-chain of fibrinogen interacts with the fibrinogen receptor on activated platelets. The data also support the concept that the sequence Arg-Gly-Asp-Ser has been conserved for use in a variety of cellular adhesive processes.

EXPERIMENTAL PROCEDURES

Peptide Preparation—The peptides Arg-Gly-Asp-Ser and Arg-Gly-Try-Ser-Leu-Gly were prepared by Peninsula Laboratories, Belmont, CA. The preparations were homogeneous when analyzed by high performance liquid and thin layer chromatography. Amino acid analysis of an acyl hydrolysate of the peptides was also consistent with their predicted composition. Prior to use, the peptides were dissolved at a concentration of 5 mg/ml in a buffer consisting of 150 mM NaCl, 50 mM sodium phosphate, pH 7.4.

Platelet Preparation—Platelet-rich plasma was obtained by differential centrifugation at 25°C of fresh, whole human blood anticoagulated with 0.1 volume of 0.13 M sodium citrate. For studies using gel-filtered platelets, 4-ml aliquots of the platelet-rich plasma were applied to 40-ml columns of Sepharose 2B (Pharmacia) equilibrated with an elution buffer containing 137 mM NaCl, 5.6 mM glucose, 0.35 mg/ml bovine serum albumin (Fraction V, Sigma), 3.3 mM NaH2PO4, and 4 mM Hepes. Fraction containing the highest platelet concentrations were pooled and the platelet count was determined with a Coulter Model ZB particle counter.

Studies of Platelet Function—Platelet aggregation studies were performed in a Chrono-log Aggregometer at 37°C (13). For aggregation studies using platelet-rich plasma, aliquots of the peptide solutions were added directly to 0.5 ml of platelet-rich plasma 5 min prior to platelet stimulation. For aggregation studies using gel-filtered platelets, 200 μg/ml fibrinogen (Kabi) and 0.5 mM CaCl2 were added to the platelet suspensions. Both fibrinogen and CaCl2 were omitted from the reaction mixtures when thrombin was the platelet agonist. Thrombin-stimulated platelet secretion was studied using platelets preloaded with 125I-fibrinogen and 0.5 mM CaCl2. The platelet-rich plasma was incubated with 0.2 μCi/ml [125I]serotonin (New England Nuclear) for 30 min at 25°C and then gel-filtered. Aliquots (300 μl) of the labeled platelet suspensions were incubated for 3 min at 37°C in the presence or absence of the peptides before α-thrombin (Parke-Davis) was added. After an additional 5 min, the incubations were stopped by sedimenting the platelets through a mixture of silicone oil (Biphenyl silicone/Methyl silicone, 4:1, William F. Nye, Inc., Fairhaven, MA). The quantity of [125I]serotonin secreted by the stimulated platelets was calculated after counting a portion of the supernatant buffer for 12C. Platelet shape change was evaluated by examining the platelet aggregation tracings and by observing the platelets with a phase contrast microscope.

Measurement of Fibrinogen Binding to ADP-stimulated Platelets—Fibrinogen binding to ADP-stimulated platelets was measured as described previously (6). Briefly, purified human fibrinogen was radiolabeled with 125I by the chloramine-T technique (14). Suspensions of gel-filtered platelets (105/ ml) were mixed with various concentrations of the 125I-fibrinogen and 0.5 mM CaCl2. To initiate fibrinogen binding, the platelets were stimulated with ADP and were incubated at 37°C for 3 min without stirring. To terminate

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Plasma and cell surface fibronectins are glycoproteins that mediate the attachment of cells to extracellular matrices (1). Recently Pierschbacher and Ruosland (2) demonstrated that a peptide with the sequence Arg-Gly-Asp-Ser has the cell attachment function of plasma fibronectin. This finding has been supported by observations of Yamada and Kennedy (3).
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the binding reaction, the platelets were sediments through silicone oil in an Eppendorf centrifuge. The tips of the centrifuge tubes containing the pelleted platelets were cut off and counted for \(^{125}\)I. Fibrinogen non-specifically bound to the pelleted platelets was measured by performing the fibrinogen binding assays in the presence of a 15-fold excess of unlabeled fibrinogen. Non-specific binding represented 10% or less of the total \(^{125}\)I-fibrinogen associated with the stimulated platelets. The effect of the peptides on fibrinogen binding was measured by adding the dissolved peptides to the platelet suspensions 3 min prior to the addition of ADP. Analysis of the binding data was aided by the use of a Texas Instruments TI programmable 58 calculator.

RESULTS

The effect of the tetrapeptide Arg-Gly-Asp-Ser on platelet aggregation was examined in both platelet-rich plasma and suspensions of gel-filtered platelets. In platelet-rich plasma, 100–300 \(\mu\)M Arg-Gly-Asp-Ser inhibited platelet aggregation stimulated by ADP, collagen, and \(\gamma\)-thrombin. As seen in Fig. 1A, 120 \(\mu\)M Arg-Gly-Asp-Ser inhibited both primary and secondary platelet aggregation stimulated by 2 \(\mu\)M ADP. Identical concentrations of the peptide Arg-Gly-Tyr-Ser-Leu-Gly did not demonstrate this effect. Because the addition of approximately a 3-fold excess of exogenous human fibrinogen to the platelet-rich plasma, but not the addition of an equivalent concentration of the protein casein, nearly reversed the inhibitory effect of Arg-Gly-Asp-Ser, it seemed likely that the tetrapeptide was interfering with the ability of fibrinogen to bind to the platelet fibrinogen receptor. However, the plasma concentration of fibrinogen (\(\approx 9 \mu\)M) is nearly 90-fold greater than the dissociation constant of the fibrinogen receptor (0.1 \(\mu\)M) (6). To study the effect of Arg-Gly-Asp-Ser at fibrinogen concentrations nearer the \(K_d\) of the fibrinogen receptor, the aggregation studies were repeated using gel-filtered platelets suspended in a buffer containing 0.6 \(\mu\)M fibrinogen. Under these conditions, Arg-Gly-Asp-Ser was a more potent inhibitor of aggregation (Fig. 1B). Platelet aggregation was progressively inhibited as the concentration of the tetrapeptide was increased. Fifty per cent inhibition occurred at Arg-Gly-Asp-Ser concentrations of 60–80 \(\mu\)M and aggregation was largely prevented at 100–200 \(\mu\)M.

Because the effect of Arg-Gly-Asp-Ser on platelet aggregation might have been due to interference with platelet activation, we examined the effect of the tetrapeptide on three other manifestations of platelet activation: shape change, secretion, and prostaglandin synthesis. The inhibition of aggregation was not associated with an inhibition of platelet shape change (Fig. 1B), a finding confirmed by observing the activated platelets with a microscope. Platelet secretion was measured as thrombin-stimulated \([^{14}\text{C}]\text{serotonin secretion because it does not depend on prior platelet aggregation} (15).

As seen in Table I, 230 \(\mu\)M Arg-Gly-Asp-Ser had no effect on thrombin-stimulated platelet \([^{14}\text{C}]\text{serotonin secretion. Finally, the inhibitory effect of Arg-Gly-Asp-Ser did not depend on platelet prostaglandin synthesis because the tetrapeptide still inhibited primary aggregation in the presence of cyclooxygenase inhibitor indomethacin (data not shown). Thus, Arg-Gly-Asp-Ser appears to be a specific inhibitor of the aggregation phase of platelet function.

Fibrinogen binding to receptors on the platelet surface is a prerequisite for platelet aggregation (16). Therefore, we examined the ability of Arg-Gly-Asp-Ser to inhibit fibrinogen binding to activated platelets. The binding of \(^{125}\)I-labeled human fibrinogen to ADP-stimulated, gel-filtered human platelets was measured as a function of fibrinogen concentration both in the absence and in the presence of the tetrapeptide. As seen by the binding isotherms in Fig. 2A, Arg-Gly-Asp-Ser inhibited the specific binding of \(^{125}\)I-fibrinogen to ADP-stimulated platelets. When these data were analyzed as

![Fig. 1. Inhibition of platelet aggregation by Arg-Gly-Asp-Ser (RGDS). A, inhibitory effect of 120 \(\mu\)M Arg-Gly-Asp-Ser on the aggregation of platelets in plasma stimulated by 2 \(\mu\)M ADP. In the middle tracing, exogenous fibrinogen was added raising the final fibrinogen concentration to 17.6 \(\mu\)M. At this fibrinogen concentration and in the absence of Arg-Gly-Asp-Ser, platelet aggregation proceeded normally. B, inhibitory effect of Arg-Gly-Asp-Ser on the aggregation of ADP-stimulated gel-filtered platelets. Platelets were gel-filtered as described under "Experimental Procedures" and 0.5 mM CaCl\(_2\) and purified human fibrinogen (final concentration, 200 \(\mu\)g/ml or 0.6 \(\mu\)M) were added to the suspensions. Platelet aggregation was stimulated by 19 \(\mu\)M ADP in the presence or absence of various concentrations of Arg-Gly-Asp-Ser. Platelet aggregation in the absence of the tetrapeptide was designated 100% aggregation.

| Thrombin concentration (\(\mu\)M) | Control | 230 \(\mu\)M Arg-Gly-Asp-Ser |
|----------------------------------|---------|-----------------------------|
| units/ml                         | %       | %                           |
| 0.01                             | 12      | 12                          |
| 0.03                             | 29      | 49                          |
| 0.05                             | 36      | 54                          |
| 0.10                             | 62      | 68                          |
| 1.00                             | 82      | 89                          |
Inhibition of Platelet Aggregation and Fibrinogen Binding

Inhibition of $^{125}$I-fibrinogen binding to ADP-stimulated platelets by Arg-Gly-Asp-Ser (RGDS). Various concentrations of $^{125}$I-labeled human fibrinogen were incubated with suspensions of gel-filtered human platelets (10$^6$ platelets/ml) at 37°C in the presence or absence of Arg-Gly-Asp-Ser. The platelets were then stimulated with 10 μM ADP. After 3 min, specific fibrinogen binding was measured as described under “Experimental Procedures.” A, binding isotherms: specific $^{125}$I-fibrinogen binding measured in the absence of Arg-Gly-Asp-Ser (○) or in the presence of 23 μM (○) and 69 μM (△) Arg-Gly-Asp-Ser. B, double reciprocal plots of the data in A.

Fig. 3. Effect of Arg-Gly-Asp-Ser (RGDS) concentration on $^{125}$I-fibrinogen binding to ADP-stimulated platelets. A, aliquots of a suspension of gel-filtered human platelets (10$^6$ platelets/ml) were incubated with either 180 μg/ml $^{125}$I-fibrinogen (○) or 48 μg/ml $^{125}$I-fibrinogen (○) and various concentrations of Arg-Gly-Asp-Ser at 37°C. The platelets were stimulated with 10 μM ADP, and $^{125}$I-fibrinogen binding to these platelets was measured as described under “Experimental Procedures.” B, analysis of the binding data in A using Dixon plots.

Discussion

The tetrapeptide Arg-Gly-Asp-Ser inhibits cellular adhesion mediated by fibronectin and also appears to be a specific inhibitor of platelet aggregation. Although it is possible that Arg-Gly-Asp-Ser exerts its effect on platelets by preventing platelet activation, this is unlikely because other platelet functions that require agonist stimulation, such as platelet shape change and platelet secretion, proceed normally in the presence of the tetrapeptide. Also, the inhibitory effect of the tetrapeptide is not due to inhibition of platelet prostaglandin synthesis because Arg-Gly-Asp-Ser was still active in the presence of the cyclooxygenase inhibitor indomethacin. However, the possibility remains that the effect of Arg-Gly-Asp-Ser does not depend upon its unique amino acid sequence, but rather upon the relatively high concentrations of tetrapeptide required to demonstrate the effect. Again, this is unlikely because equivalent concentrations of the peptide Arg-Gly-Tyr-Ser-Leu-Gly did not affect platelet function.

Arg-Gly-Asp-Ser appears to exert its effect on platelet
aggregation by inhibiting fibrinogen binding to activated platelets. Fibrinogen binds to receptors on the platelet surface that are exposed by platelet activation. This receptor-bound fibrinogen promotes platelet aggregation, perhaps by cross-linking adjacent platelets. The inhibitory effect of Arg-Gly-Asp-Ser on fibrinogen binding is complex. Analysis of our fibrinogen binding data on double-reciprocal and Dixon plots indicated that Arg-Gly-Asp-Ser is a competitive inhibitor. Thus, Arg-Gly-Asp-Ser decreases the affinity of the available fibrinogen receptors without altering their number. However, higher Arg-Gly-Asp-Ser concentrations did not completely inhibit fibrinogen binding, indicating that the tetrapeptide is a partial inhibitor (18) and that fibrinogen receptors containing the tetrapeptide can still interact with fibrinogen to some extent but with a decreased affinity. Other data demonstrating that portions of the fibrinogen γ-chain also interact with the fibrinogen receptor are consistent with this interpretation. Kloczewiak and co-workers (9, 10) have shown that synthetic pentadecapeptide and dodecapeptide analogues of the carboxyl-terminus of the fibrinogen γ-chain inhibit platelet aggregation and fibrinogen binding with an IC₅₀ of 28 μM, a value similar to the K₅₀ of Arg-Gly-Asp-Ser. The fact that high concentrations of either the α or the γ peptide analogues are required to inhibit fibrinogen binding and platelet aggregation also suggests that other factors, such as the three-dimensional structure of the intact fibrinogen molecule, are important in fibrinogen binding to its platelet receptor.

Our conclusion that a segment near the carboxyl-terminus of the fibrinogen α-chain is involved in fibrinogen binding to the fibrinogen receptor is supported by other evidence in the literature. For example, Kloczewiak and co-workers demonstrated that polymerized fibrinogen α-chains support platelet aggregation, although not as well as polymerized γ-chains (11). Earlier studies by Niewiarowski et al. (12) had also shown that plasmin-generated fragments of fibrinogen lacking intact carboxyl-termini of the α-chains were up to 8-10 times less potent in supporting platelet aggregation than intact fibrinogen.

The peptide sequence we have studied is present as residues 572 through 575 near the carboxyl-terminus of the 610 residue fibrinogen α-chain (19). This portion of the α-chain extends as a highly polar appendage from each end of the fibrinogen molecule (19) and, therefore, is available to bind to exposed fibrinogen receptors on adjacent platelets. The tetrapeptide sequence is also present in fibronectin, most likely in a hydrophilic loop on the surface of the molecule (2). Of considerable interest, Ginsberg and his colleagues (20–22) have reported that the tetrapeptide Arg-Gly-Asp-Ser inhibits fibronectin binding to activated platelets. Thus, it appears that the tetrapeptide sequence we have studied is involved in the interaction of both fibrinogen and fibronectin with the platelet surface. Moreover, because the amino acid sequence Arg-Gly-Asp-Ser is involved in adhesive reactions mediated by two different macromolecules and can be found on at least five other proteins such as the λ phage receptor of Escherichia coli and the Sindbis virus coat protein, our data lend support to the concept that this amino acid sequence has been conserved to support cellular adhesion in a variety of biological processes (23).

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