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COMMENTARY

FIBRINOGEN, FIBRINOGEN RECEPTORS, AND THE PEPTIDES THAT INHIBIT THESE INTERACTIONS

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In the events beginning with fertilization and extending through embryogenesis, organ development and growth and in the host defense mechanisms essential for survival, cell–cell and cell–substratum interactions are crucially involved. Such cellular adhesive reactions are frequently governed by a common mechanism: the recognition of extracellular ligands in substrata or on other cells by cell surface receptors. This basic mechanism is observed in the cellular responses of the simplest of multicellular organisms and extends to the most highly specialized adhesive reactions of mammalial cells. Platelets, anucleated cell fragments which circulate in human blood at approximately 5×10^8 per ml, perform the adhesive reactions necessary for protection of the organism from excessive bleeding at sites of vascular injury. As aberrations of platelet adhesive functions can result in thrombotic diseases or bleeding, the adhesive reactions of platelets have been investigated intensively. Moreover, with their simplicity, particularly in terms of their membrane protein composition, their ease of isolation at high yield, and the facile measurement of their adhesive responses, the platelet is accepted as an excellent model for analyses of cellular adhesive reactions. The particular relevance of platelets as model cells has been further emphasized by the recent demonstration that a variety of nucleated cells possess membrane proteins that are structurally and immunologically related to the platelet membrane proteins involved in adhesive reactions [1, 2].

In this commentary, we shall briefly summarize how the general mechanism of receptor-mediated recognition of extracellular ligands applies to platelet adhesive reactions. A focus of recent studies in this area has been the recognition specificities of the platelet receptors for the extracellular ligands. These studies have led to the identification of three structurally distinct peptide sets that inhibit platelet adhesive reactions by interfering with the interaction of a family of extracellular ligands, which includes fibrinogen (Fg), with its receptors. The primary emphasis of this commentary will be on these three peptide sets and the basis for their inhibitory activity. As these peptides are homologous to primary amino acid sequences in Fg and as they are structurally dissimilar to one another, it is attractive to hypothesize that each peptide defines a sequence in Fg that interacts with the platelet receptor for Fg. We shall consider this hypothesis and summarize evidence to indicate that this interpretation does not apply to at least one of the peptide sets. Furthermore, we will emphasize the broad gap between existing experimental data and the premise that the other two peptide sets define distinct recognition specificities of the platelet receptors for Fg. Thus, we will call for a more cautious interpretation of existing data and identify specific experimental questions that need to be addressed to clarify the mechanisms by which these peptides act as antagonists of platelet adhesive reactions.

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PLATELET ADHESIVE PROTEINS AND THEIR RECEPTORS

Over the past two decades, three broad sets of observations have provided key insights into the mechanisms underlying platelet adhesive reactions. First, a family of proteins which can serve as extracellular ligands for mediating or modulating platelet adhesive reactions has been identified (reviewed in Ref. 3. For brevity, reviews are cited where possible.). This adhesive protein family includes Fg, fibronectin (Fn), and von Willebrand factor (vWF). Fg plays a predominant role in platelet aggregation, whereas Fn and vWF are more centrally involved in platelet substratum linkages. Second, specific membrane proteins that participate in platelet adhesive reactions have been identified. GPIb is involved in platelet adhesion to substratum, whereas GPIIb-IIIa participates in the platelet–platelet contact. These assignments are, in part, based upon the abnormalities associated with genetic deficiencies of GPIb in Bernard–Soulier Syndrome and of GPIIb-IIIa in Glanzmann's thrombasthenia (reviewed in Ref. 4), and they are not absolute as GPIIb-IIIa has been implicated in platelet adhesion to collagen and subendothelium [5–7]. Third, a more recent development and one which links the first two observations—GPIb and GPIIb-IIIa serve as receptors for the adhesive proteins. GPIb binds vWF, whereas GPIIb-IIIa binds Fg, Fn and vWF (reviewed in Refs. 8 and 9).

GPIIb-IIIa is a member of the cytoadhesin family, a group of immunologically and structurally related

cell surface molecules which share a functional role in cellular adhesive reactions. The vitronectin receptor and molecules found on the surface of endothelial cells, fibroblasts, smooth muscle cells, certain monocytoid cells and megakaryocytes are members of the cytoadhesin family [1, 2]. The cytoadhesins are also related to other families of cell surface molecules such as the leukocyte adhesion molecules of lymphocytes (LFA-I), monocytes (Mac-I) and neutrophils (p150,95) [10] and the family of 140 kDa Fn receptors [11] that includes the chicken fibroblast Fn receptor, integrin [12]. GPIIb has a molecular weight of ~132,000 and is comprised of a heavy and light chain. GPIIIa is a single subunit of ~100,000 molecular weight. Upon reduction, the apparent size of GPIIIa on sodium dodecyl sulfate (SDS)-polyacrylamide gels increases, reflecting its high content of intrachain disulfide bonds. This property is common to the beta (GPIIIa-related) subunit of most members of the cytoadhesin family. GPIIb-IIIa exists on the platelet surface as a noncovalent heterodimeric complex of its GPIIb and GPIIIa subunits. Calcium plays an essential role in maintaining the subunits in the complexed state (reviewed in Ref. 4).

Despite the availability of micromolar concentrations of the adhesive proteins in plasma, GPIIb-IIIa is unoccupied on the platelet surface. Cellular activation is necessary to render GPIIb-IIIa competent for binding functions. A number of physiologic and pharmacologic stimuli including ADP, epinephrine, thrombin, collagen and calcium ionophore alter platelets such that GPIIb-IIIa acquires receptor binding properties. The repertoire of stimuli that support Fg and vWF binding to GPIIb-IIIa is probably similar, whereas agonists which support Fn binding are more restricted [3, 8]. The mechanisms by which platelet stimulation converts GPIIb-IIIa to a competent state remain to be established. Evidence has been presented to indicate that many of the intracellular signaling mechanisms associated with stimulus–response coupling in other cells are activated upon platelet stimulation, but convincing cause and effect relationships with respect to rendering GPIIb-IIIa a functional receptor have yet to be demonstrated.

PEPTIDES AND THE RECOGNITION SPECIFICITIES OF THE FIBRINOGEN RECEPTOR

Fg, Fn and vWF are large glycoproteins with molecular weights in excess of 340,000 so that assignment of platelet binding to discrete segments of the adhesive proteins has been challenging. Nevertheless, small proteolytic fragments have been identified within each adhesive protein which retain the binding properties of the parent molecules [13–15]. Furthermore, small peptides have been synthesized which correspond to specific primary amino acid sequences within the adhesive proteins, and these peptides inhibit binding and platelet adhesive responses. Thus far, three structurally unrelated peptide sets which inhibit Fg binding to platelets have been identified. Table 1 describes these three peptide sets. For simplicity and because there is some latitude with respect to permissive amino acid substitutions with retention of biological activity, the peptide sets will be referred to as the Gly-Pro-Arg-Pro, Arg-Gly-Asp, and the gamma chain peptides. These peptides correspond in structure to primary amino acid sequences that are either present or are closely homologous to sequences in Fg. Sequences corresponding to two of the peptides, Gly-Pro-Arg-Pro and the gamma chain peptides, are found in Fg but not Fn or vWF, whereas Arg-Gly-Asp is found within Fn and vWF and is present in two locations in Fg. Nevertheless, the gamma chain peptides as well as Arg-Gly-Asp peptides inhibit the binding of all three adhesive proteins to platelets, and only Gly-Pro-Arg-Pro peptides selectively inhibit Fg binding to platelets. To facilitate the localization of the internal sequences in Fg that correspond to the peptide sets, a simple schematic model of Fg has been depicted in Fig. 1. This model illustrates the three pairs of constituent chains, Aα, Bβ and γ chains, of Fg. The organization of the molecule into its domainal structure is also shown. It is comprised of a central E domain, which contains the amino termini of all six polypeptide chains. This domain contains the two sets of sequences homologous to Gly-Pro-Arg-Pro and one of the two sets of Arg-Gly-Asp sequences. The two peripheral D domains contain portions of the Aα, Bβ and γ chains, including the carboxy terminal of the γ chain. The carboxy terminal two-thirds of each Aα chain is regarded as a separate hydrophilic domain, and these segments also contain an Arg-Gly-Asp sequence. Each of the three peptide sets is discussed individually below in terms of its mechanism of action.

Gly-Pro-Arg-Pro peptides. Gly-Pro-Arg-Pro was the first of the three peptide sets shown to inhibit Fg binding to platelets and platelet aggregation [16]. As illustrated in Fig. 2A, Gly-Pro-Arg-Pro produces a dose-dependent inhibition of Fg binding to thrombin-stimulated platelets. At the concentration of the radiolabeled Fg used, the ID_{50} of Gly-Pro-Arg-

Table 1. Peptides which inhibit fibrinogen binding to platelets

Peptide set	Peptide structure	Sequence in fibrinogen	Effect on adhesive protein–platelet interaction
1. Gly-Pro-Arg-Pro	GPRP	Homologous to Aα 17–20 Bβ 15–18	Selective inhibition of Fg binding
2. Arg-Gly-Asp	RGD	Aα 95–97 Aα 572–574	Inhibition of Fg, Fn and vWF
3. γ Chain peptides	LGGAKQAGDV	γ 402–411	Inhibition of Fg, Fn and vWF

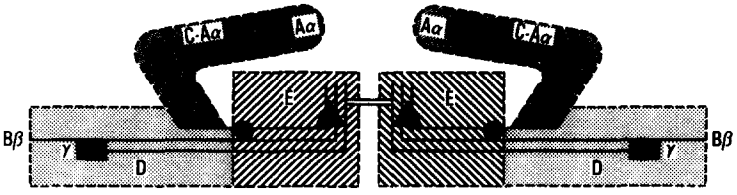

Fig. 1. Schematic representation of the fibrinogen molecule depicting the location of the active peptide sets that inhibit fibrogen binding to platelets. Sequences homologous to Gly-Pro-Arg-Pro are indicated by (▲); RGD sequences are indicated by (●); and the gamma chain sequence is indicated by (■). The domains of fibrinogen are depicted by the different shadings.

Pro, the concentration of the peptide producing 50% inhibition of Fg binding, was 70 μM; the maximum inhibition of Fg binding at high Gly-Pro-Arg-Pro concentrations (700 μM) was approximately 65%; and no inhibition of Fn or vWF binding was observed. The peptide inhibited the extent of Fg binding to ADP and thrombin-stimulated platelets under equilibrium conditions in the presence of either calcium or magnesium. Consistent with the capacity of Gly-Pro-Arg-Pro to inhibit Fg binding, the peptide also inhibited platelet aggregation. It is noteworthy that Gly-Pro-Arg-Pro completely inhibits platelet aggregation at concentrations at which Fg binding was only 50% suppressed, suggesting that receptor occupancy may not be sufficient to support the functional response. Other independent lines of evidence also support this conclusion. Using ADP-fixed platelets, cells stimulated with an agonist such as ADP and then fixed with paraformaldehyde, Gly-Pro-Arg-Pro maintained its capacity to inhibit Fg binding although its activity was diminished as compared to "living" platelets. This suggests that Gly-Pro-Arg-Pro can directly interfere with Fg binding to its receptor and may also exert some effect on receptor induction.

When thrombin converts human Fg to fibrin, it cleaves a 16 amino acid peptide, fibrinopeptide A, from the amino terminus of the Aα chain of Fg and a 14 amino acid peptide, fibrinopeptide B, from the amino terminus of the Bβ chain of Fg to generate new amino termini at the α and β chains of fibrin. Gly-Pro-Arg-Pro is homologous to the amino termini of the α chain, Gly-Pro-Arg-Val-Val, and the β chain, Gly-His-Arg-Pro. Gly-Pro-Arg-Val-Val inhibited Fg binding to platelets but was less active than Gly-Pro-Arg-Pro. Gly-His-Arg-Pro was still less inhibitory. Methylation of the amino terminus of Gly-Pro-Arg-Pro completely abrogated its inhibitory activity. These results indicated a fine structural specificity for the inhibitory effects of this peptide set, a conclusion which is applicable to all three peptide sets.

A telling experiment into the mechanism of action of Gly-Pro-Arg-Pro was provided by examining the effect of Fg concentration on the inhibitory activity of the peptide. With Gly-Pro-Arg-Pro present at a high concentration, minimal inhibition of binding was observed at input Fg concentrations at or below its K_d (0.3 μM). At higher concentrations of Fg, inhibition increased with increasing Fg concentrations. This behavior differs from that of the other two peptide sets discussed below and is consistent with the following hypothesis: Gly-Pro-Arg-Pro binds with relatively low affinity to Fg, and this complex no longer binds to platelets. Accordingly, as the concentration of Fg increases, the concentration of the Fg-Gly-Pro-Arg-Pro complex also increases. The peptide binds only to Fg (or if it binds to Fn and vWF, it binds to sites which are not involved in platelet recognition), and this explains the selectivity of Gly-Pro-Arg-Pro for inhibition of Fg binding. This interpretation is compatible with the demonstrated capacity of Gly-Pro-Arg-Pro to bind to Fg. Gly-Pro-Arg-Pro was originally identified as an inhibitor of fibrin polymerization. This anti-polymerization activity is depen-

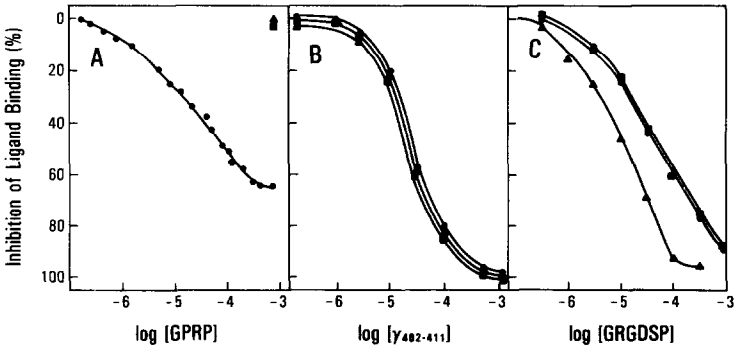

Fig. 2. Effects of the three peptide sets on the binding of adhesive proteins to thrombin-stimulated platelets. [^{125}I]Fibrinogen (●), [^{125}I]fibronectin (▲) and [^{125}I]von Willebrand Factor (■) were added to thrombin-stimulated platelets in the presence of various concentrations of the indicated peptides. Results are taken from Refs. 16, 19 and 27.

dent upon the capacity of this peptide to mimic sites in the E domain and bind to sites in the D domain of Fg [17]. This same interaction may also interfere with Fg binding to platelets. The effective concentrations of Gly-Pro-Arg-Pro for inhibiting Fg binding to platelets are consistent with the K_d estimated for its binding to the D domain of Fg. The null hypothesis that Gly-Pro-Arg-Pro binds directly to platelets and, thereby, inhibits Fg binding lacks support as no direct binding of a radiolabeled Gly-Pro-Arg-Pro derivative to platelets was demonstrable. Thus, it seems likely that the sequences in Fg homologous to Gly-Pro-Arg-Pro are not directly involved in Fg binding to platelets, and the peptide set falls into the category of antagonists which bind to ligands and prevent their interaction with their receptors.

Gamma chain peptides. Peptides corresponding in sequence to the extreme carboxy terminus of the γ chain of Fg inhibit Fg binding to stimulated platelets [18, 19]. The decapeptide used in Fig. 2B has the structure of LGGAKQAGDV, which corresponds to γ 402–411 of the 411 amino acid γ chain. It inhibits Fg binding in a dose-dependent manner with an ID_{50} of 50–100 μM and a maximal inhibition of >90%. This peptide also inhibits Fn and vWF binding to thrombin-stimulated platelets with inhibition curves that are virtually superimposable. Additional studies of the gamma chain peptide set have indicated that it inhibits the extent of Fg binding at equilibrium and that it is equally potent in the presence of calcium and magnesium. The gamma chain peptides inhibit Fg as well as Fn and vWF binding to stimulated-fixed platelets, indicating that they interfere directly with the ligand binding interaction. Finally, a fine structural specificity for the inhibitory activity of this peptide set has been demonstrated, as the lysine (K) and the carboxy terminal Asp-Val (DV) residues are very important for inhibitory activity. The gamma chain peptides can be extended at their amino terminal end with sequences corresponding to the γ chain of Fg without loss of biological activity. Whether addition of 1 to 5 amino acids to the amino terminus of the decapeptide appreciably enhances inhibitory potency is variable in different laboratories [18, 19].

The inhibition of Fg binding to platelets by the gamma chain peptides is presumed to arise from competition between the free peptides and the γ chain sequence within Fg for the platelet receptor. Several lines of evidence support this supposition. (1) Large proteolytic fragments of Fg, which contain the carboxy terminus of the γ chain, inhibit Fg binding to platelets. When this segment of the γ chain is removed from these fragments by further proteolysis, inhibitory activity is lost [20]. (2) Free γ chains interact with platelets, and antibodies to the γ chain inhibit Fg binding to the cells [21]. (3) Certain heterobifunctional reagents crosslink the γ chain of Fg to GPIIb-IIIa [22]. (4) Fg with an alternative sequence at its carboxy terminal aspect of its γ chain, the γ' chain, binds poorly to platelets [23]. (5) Based on double-reciprocal analyses, the gamma chain peptides inhibit Fg binding to platelets by a mechanism consistent with competitive antagonism [19]. (6) The gamma chain peptides appear to bind directly to platelets [18]. (7) The gamma chain peptides, coupled to a solid support, selectively and specifically bind GPIIb-IIIa from detergent extracts of whole platelets [24].

Despite the preponderance of the above-cited evidence, we contend that it is still premature to assign a platelet recognition site to the carboxy terminal of the γ chain. Our concern is based upon two critical issues. First, it must be demonstrated that the gamma chain peptides bind to platelets with a stoichiometry and affinity consistent with their proposed function. Therefore, the number of gamma chain binding sites must approximate the number of platelet receptors for Fg, and its K_d should be similar to its K_i for inhibition of Fg binding to platelets. Such data may be forthcoming but are not available at present. Second, and what must be regarded as a more disconcerting point, an explanation must be provided for the capacity of the gamma chain peptides to also inhibit Fn and vWF binding to platelets. There are no clear sequence homologies between the gamma chain peptides and the primary sequences of Fn and vWF. Thus, competition between the free peptides and internal protein sequences may not be the basis for the inhibitory activity of the gamma chain peptides. Until this point is reconciled, it would seem rather arbitrary to accept one mechanism for inhibition of Fg binding by the gamma chain peptides which does not also apply to Fn and vWF.

Arg-Gly-Asp peptides. The Arg-Gly-Asp sequence was originally identified as a site within Fn which expresses cell attachment activity [25]. Arg-Gly-Asp peptides dissociated fibroblasts from Fn substratum, and these cells could adhere directly to the peptides [26]. The binding of Fn to platelets provided a means to quantitatively assess the effects of these Arg-Gly-Asp peptides on the direct interaction of Fn with a cellular receptor, and it was demonstrated that micromolar concentrations of these peptides inhibited this interaction as well as platelet aggregation with the same structural specificity as observed for fibroblasts [14]. Subsequently, it was shown, as illustrated in Fig. 2C, that the Arg-Gly-Asp peptides also inhibit Fg and vWF binding to stimulated platelets [27–29]. It is noteworthy that the particular Arg-Gly-Asp peptide used in Fig. 2C, Gly-Arg-Gly-Asp-Ser-Pro, was more effective in inhibiting Fn than Fg and vWF binding. The Arg-Gly-Asp peptides share virtually all the same characteristics with the gamma chain peptides with respect to their pattern of inhibition of Fg binding. Namely, these peptides inhibit the extent of Fg binding at equilibrium; they are effective in the presence of either calcium or magnesium; they inhibit ligand binding to stimulated-fixed platelets; and they exhibit a fine structural specificity to their inhibitory activity. The final point is illustrated by the fact that conservative amino acid substitutions for the Arg, Gly or Asp markedly diminish inhibitory potency [27].

As with the gamma chain peptides, the existence of an Arg-Gly-Asp recognition site which mediates Fg binding to platelets can also be supported by a series of persuasive evidence. (1) Proteolytic fragments of Fg which lack the carboxy terminal region of the Aα chain containing the Arg-Gly-Asp sequence support platelet aggregation less well than intact Fg [30]. (2) Isolated Aα chains bind to platelets and cause their aggregation [21]. (3) The crosslinking of Fg to

platelets by certain heterobifunctional reagents occurs via the Aα chain [31]. (4) Arg-Gly-Asp peptides behave as apparent competitive antagonists of adhesive protein (Fn) binding to platelets based upon double-reciprocal analyses [14]. (5) Arg-Gly-Asp peptides bind directly to platelets [32]. (6) Arg-Gly-Asp coupled to a solid support selectively binds GPIIb-IIIa from detergent extracts of platelets, and the free peptide elutes GPIIb-IIIa from the resin [33].

Two concerns were cited above which precluded interpreting a similar body of evidence as defining a platelet recognition specificity for the γ chain of Fg. These concerns do not apply to the Arg-Gly-Asp peptide set. (1) The direct interaction of an Arg-Gly-Asp peptide with platelets has been measured in our laboratory. The K_d of this interaction is consistent with the K_i of this peptide on Fn binding to platelets [22]. (2) As all three adhesive proteins contain an Arg-Gly-Asp sequence, the capacity of this peptide set to inhibit the binding of these three adhesive proteins to platelets could be explained in terms of a competition between the free peptides and the internal sequences within the proteins. However, other substantial concerns arise with the Arg-Gly-Asp peptides. First, as noted above, certain Arg-Gly-Asp peptides are of unequal potency in inhibiting Fg, Fn and vWF binding [27]. If a peptide interacts with the platelet by a single binding mechanism, its inhibitory potency should be the same for all three adhesive proteins. This indicates that a more complex mechanism of inhibition may be involved. Second, Fg derivatives lacking the Arg-Gly-Asp sequence in the carboxy terminal aspects of the Aα chain are reported to bind to platelets with an affinity similar to intact Fg [34]. This observation can be rationalized as implicating the more amino terminal Arg-Gly-Asp sequence at Aα 95–97 in Fg binding to platelets, but it also may indicate that not all Arg-Gly-Asp sequences in proteins can interact with Arg-Gly-Asp receptors. If this is the case, then conclusive evidence that the Aα 95–97 is involved in Fg binding must be provided. Third, we have found that γ chain peptides elute GPIIb-IIIa from Arg-Gly-Asp affinity columns and the γ chain peptides inhibit the binding of Arg-Gly-Asp peptides directly to platelets [24]. This is observed despite the lack of apparent sequence homology between the two peptide sets. (AGDV, the carboxy terminal sequence of the gamma chain peptides, is a very poor inhibitor of Fg binding.) These observations indicate that the binding sites for these two peptide sets cannot be independent from one another. Either the two peptide sets bind to the same sites or their binding sites must be closely linked in a steric or allosteric fashion. Therefore, the inhibition of Fg binding by either peptide set could be due to an effect of the peptides on the recognition of the gamma chain or the Arg-Gly-Asp sequences in Fg.

CONCLUDING REMARKS

In view of these substantial concerns regarding the role of both the γ chain and the Arg-Gly-Asp sequences, it seems premature to assign a precise recognition specificity(ies) to the platelet receptor for Fg. While it is now clear that both of these peptide sets can interact with platelets and apparently both can interact with GPIIb-IIIa, their sites of interaction are not independent and may be identical or mutually exclusive. More importantly, it is not clear that the corresponding sequences in Fg interact with this peptide binding site(s). The relatively low affinity of the platelet receptors for the adhesive proteins and the even lower affinities of the platelet binding sites for the peptides complicates the resolution of these issues. The possible existence of, as of yet, unidentified additional subsites within the adhesive proteins which contribute to the measured interactions also complicates the resolution of these questions. Creative and independent approaches may be necessary in order to determine the recognition specificities of the platelet receptors for Fg and the adhesive proteins and to precisely define the contribution of the gamma chain and the Arg-Gly-Asp sequences to Fg binding to platelets.

Finally, while the mechanism of inhibition of Fg binding by these peptide sets remains unresolved, this does not preclude a brief consideration of their utility as anti-thrombotic agents. Agents that inhibit specific platelet adhesive reactions without causing irreversible paralysis of other platelet functions would seem to be highly advantageous. Drugs based on any of the three peptide sets could selectively inhibit platelet aggregation without interfering with other platelet functions or adhesive reactions independent of GPIIb-IIIa mediated events. Moreover, since such drugs could be potentially short-acting in that their removal could reverse the inhibition of platelet aggregation, it should be possible to create a temporary anti-thrombotic state. Gly-Pro-Arg-Pro related structures are intriguing in this regard in that they would inhibit fibrin polymerization as well as platelet aggregation and, thereby, inhibit both limbs of the thrombus formation pathways. The large sink of plasma Fg would mean that very high concentrations of Gly-Pro-Arg-Pro based structures would have to be administered unless much higher affinity analogs were created. With the γ chain and the Arg-Gly-Asp peptides, cellular selectivity would be a major issue. Arg-Gly-Asp peptides affect the adhesive properties of a variety of cells. Whether analogs can be designed which are selectively targeted to the platelet Arg-Gly-Asp binding sites would be a central issue. In favor of this possibility is the observation that, although Arg-Gly-Asp related peptides show the same structure–function relationships on platelet aggregation and fibroblast attachment, platelet function is considerably more sensitive to low doses of the peptides. Whether the γ chain peptides will interact with or influence the function of Arg-Gly-Asp receptors on cells other than platelets remains to be determined. Thus, the potential of these three peptide sets as anti-thrombotic agents is a provocative possibility. A clearer understanding of the mechanism of action of these peptides might provide the information necessary for a systematic rationale to the design of such selective anti-thrombotic agents based upon these peptide sequences.

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