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Binding Interactions of Kistrin With Platelet Glycoprotein IIb-IIIa: Analysis by Site-Directed Mutagenesis

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ABSTRACT **The binding interactions between platelet fibrinogen receptor, glycoprotein (GP) IIb-IIIa, and kistrin, a snake venom disintegrin protein that contains the adhesion site recognition sequence Arg-Gly-Asp (RGD) and potently inhibits platelet aggregation, have been investigated by site-directed mutagenesis of a synthetic kistrin gene. Kistrin was expressed as a fusion protein in *Escherichia coli* under control of the alkaline phosphatase promoter. This construction included the stII signal sequence to direct secretion to the periplasmic space and one synthetic (Z) domain of Staphylococcal protein A to allow affinity purification using IgG Sepharose. Kistrin was cleaved from the Z-domain by site-specific proteolysis using a mutant subtilisin BPN' and purified by reverse-phase HPLC. This approach facilitated the rapid purification of a set of 43 alanine replacement mutants whose relative affinity for GP IIb-IIIa was measured by competition with immobilized kistrin and by inhibition of platelet aggregation in human platelet-rich plasma. Alanine replacements at R49, G50, and D51 led to weaker inhibitors of platelet aggregation by 90-fold, 2-fold, and >200-fold, respectively. The conservative D51E mutant was still >100-fold less potent whereas R49K had a minor effect (1.8-fold), implying the critical nature of the aspartate for high affinity binding. However, mutations outside of the RGD region led to proteins indistinguishable from kistrin, suggesting no substantial secondary binding interactions. Furthermore, reduced kistrin is not active. We therefore propose that a favorable conformation of the RGD region alone is responsible for the high affinity binding of kistrin to GP IIb-IIIa.**

Key words: RGD sequence, snake venom disintegrin, GP IIb-IIIa antagonist, platelet aggregation inhibitor, protein structure/conformation

INTRODUCTION

Kistrin is a 68-residue protein isolated from the venom of *Agkistrodon rhodostoma* that contains 6 disulfide linkages as well as the RGD adhesion site recognition sequence.[1] It belongs to a highly homologous family of *Viperidae* snake venom proteins termed disintegrins[2] (Fig. 1) which are potent inhibitors of platelet aggregation and antagonists of glycoprotein IIb-IIIa (GP IIb-IIIa), a calcium-dependent heterodimer[3,4] belonging to the integrin family of adhesion receptors.[5] The interaction of these small, cysteine-rich, RGD-containing proteins with both purified GP IIb-IIIa and platelets has been extensively studied due to their relative abundance in snake venom, ease of purification, and their potent in vitro and in vivo effects observed with GP IIb-IIIa and platelets.[1,2] Furthermore, kistrin may have therapeutic benefits based upon its ability to accelerate the rate and extent of thrombolysis as well as prevent reocclusion in a canine model of coronary arterial thrombosis when coadministered with tissue type plasminogen activator.[6]

The conserved RGD sequence in this family and evidence that RGD-containing peptides inhibit the binding of disintegrins to platelets[7,8] indicate that the RGD sequence plays a role in binding to GP IIb-IIIa. However, kistrin and other disintegrins have much higher affinity (>100-fold) for GP IIb-IIIa or platelets than either fibrinogen (Fg) or synthetic linear RGD-containing peptides.[1,7,9] The basic question we have addressed is why kistrin is so much more potent than linear RGD-containing peptides. Two hypotheses for high affinity kistrin bind-

Abbreviations: GP, glycoprotein; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; Fg, fibrinogen; ELISA, enzyme-linked immunosorbent assay; TFA, trifluoroacetic acid; PBST, phosphate-buffered saline containing 0.01% Tween 20; IC_{50}, the protein concentration eliciting 50% of the maximal response. The one-letter abbreviation for amino acids is used to represent peptides and to identify each kistrin mutant. For example, RGD represents the peptide L-arginyl-L-glycyl-L-aspartate; the kistrin mutant R49K represents kistrin which contains L-lysine instead of L-arginine at residue 49. Double mutants are named as strings; for example, if in addition to R49K, glycine 50 was changed to L-alanine, the mutant would be called R49K/G50A.

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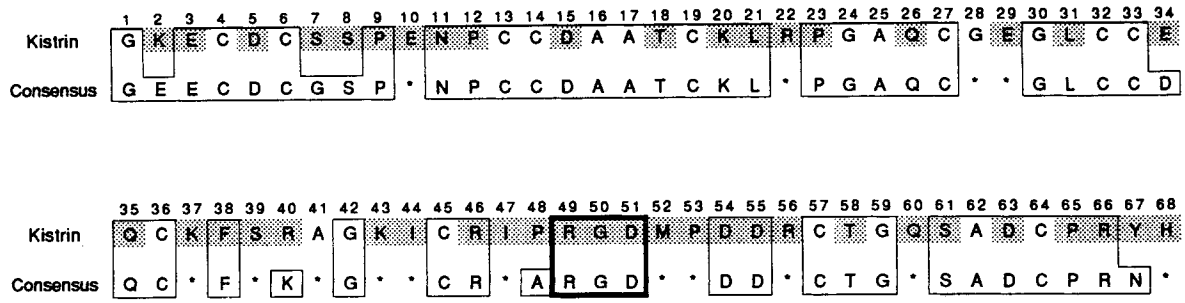

Fig. 1. Comparison of the kistrin amino acid sequence with a consensus sequence of snake venom GP IIb-IIIa antagonists. The amino acid positions are numbered across the top relative to kistrin. The consensus sequence refers to amino acids found >70% of the time in sequences published by Dennis et al.,[1] Gould et al.,[2] and Scarborough et al.[42]; identical sequences were not duplicated. Boxed positions represent amino acid identity to the consensus sequence; the RGD is boxed in bold. An asterisk is used to represent variable regions in the consensus sequence where no amino acid is found >70% of the time. Amino acids in kistrin that were changed are shaded.

ing to GP IIb-IIIa are proposed: (1) favorable conformational restraints are imposed upon the RGD sequence, which is supported by the fact that disulfide reduction of kistrin and related proteins abolishes inhibitory activity;[1,7,10] and/or (2) additional binding interaction(s) are provided by residues outside of the RGD sequence that may contribute significantly to binding. The latter is supported by the high degree of sequence homology observed in this family in addition to the RGD sequence (Fig. 1).

In this report we have used site-directed mutagenesis of kistrin to examine the relative contribution of the RGD sequence to GP IIb-IIIa binding as well as to identify any additional binding determinants outside of this region. This work was initiated in concert with an effort to determine the kistrin structure, which has recently been solved by 2D-NMR.[11] Based on the evidence from alanine replacements in the kistrin sequence and other selected mutants, we conclude that the conformation of the RGD region in the disintegrins is largely responsible for the high affinity interaction with GP IIb-IIIa.

MATERIALS AND METHODS

Materials

All enzymes for DNA manipulations were from Bethesda Research Laboratories except for *E. coli* DNA polymerase I large fragment (Klenow) and T4 DNA ligase which were obtained from Boehringer Mannheim and New England Biolabs, respectively. The isolation of snake venom-derived kistrin, fibrinogen, GP IIb- IIIa, AP3 (a murine monoclonal to human GP IIIa that does not block platelet aggregation), and other reagents for the kistrin/GP IIb-IIIa ELISA and platelet aggregation assays have been described.[1] The *E. coli* strains used were JM101 (Δ*lac-pro supE thi*{F'*tra*D36 *proAB*+ *lacI*^{Q} ZΔM5}),[12]W3110 tonA (ATCC 27325), and BMH 71-18 *mutL*.[13] Oligonucleotides were synthesized using hydrogen phosphonate chemistry[14] and purified by polyacrylamide gel electrophoresis. The pentamutant S24C/H64A/E156S/G169A/Y217L subtilisin BPN'[15] was provided by Genencor International.

Construction of a Synthetic Kistrin Gene

All DNA manipulations were performed essentially as described by Sambrook et al.[16] unless otherwise indicated. A gene for kistrin was assembled from 8 synthetic oligonucleotides ranging from 46 to 80 bases long and sharing an 18 base pair overlap with neighboring oligonucleotides (Fig. 2). Unique restriction sites were designed into the kistrin gene approximately every 30 bp to facilitate mutagenesis. The individual DNA oligonucleotides were phosphorylated, annealed together by cooling from 85 to 4°C over ca. 30 min, ligated into M13 mp18,[12] and transformed into JM101. Clones containing inserts of the correct size were sequenced by the dideoxy method of Sanger et al.[17]

Construction of the Z-Kistrin Expression Vector (pZKis)

The vector for expression of the Z-kistrin fusion protein was constructed by ligating 3 fragments: (1) 4.15 kb *NarI*/*Sty*I from pZAP-2 (kindly provided by L. Abrahmsen) containing the alkaline phosphatase promoter, stII signal sequence, one synthetic Z domain of *Staphylococcus aureus* protein A, an ampicillin resistance gene and pMB1 and f1 origins of replication; (2) *Nsi*I/blunt linker coding for cleavage by the pentamutant subtilisin, and (3) 0.22 kb *Pst*I (blunted)/*Sty*I containing the synthetic gene for kistrin from the M13 clone identified above.

Mutagenesis Procedure

In addition to the desired amino acid changes, oligonucleotides were designed to encode a silent change which created or destroyed a unique restriction site, thus allowing restriction/selection purification of mutants.[18] Site-directed mutagenesis was

A.

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 NarI                       PstI (blunted)      XhoI                                          BspMI
CG CCG GGA TTC GCG CAT TAC GGC AAA GAA TGT GAT TGC TCG AGT CCG GAA AAT CCG TGT TGT GAT GCA GCT ACC TGC AAA|
   GGC CCT AAG CGC GTA ATG CCG TTT CTT ACA CTA ACG AGC TCA GGC CTT TTA GGC ACA ACA|CTA CGT CGA TGG ACG TTT
Ala Pro Gly Phe Ala His Tyr Gly Lys Glu Cys Asp Cys Ser Ser Pro Glu Asn Pro Cys Cys Asp Ala Ala Thr Cys Lys
 Z          LINKER              KISTRIN

                                      StuI                                          BssHII              BamHI
TTG CGT CCG GGC GCT CAA TGC GGT GAA GGC CTG TGT TGT GAA CAA TGT AAA TTC TCG CGC|GCT GGT AAG ATC TGC CGG ATC
AAC GCA GGC CCG CGA GTT ACG CCA CTT CCG GAC ACA ACA CTT|GTT ACA TTT AAG AGC GCG CGA CCA TTC TAG ACG GCC TAG
Leu Arg Pro Gly Ala Gln Cys Gly Glu Gly Leu Cys Cys Glu Gln Cys Lys Phe Ser Arg Ala Gly Lys Ile Cys Arg Ile

                                 ClaI                                SacII               SphI StyI
CCT CGT GGT GAT ATG CCG GAT GAT CGA TGT ACC GGT CAA|TCT GCT GAT TGT CCG CGG TAT CAT TAA GCATGC
GGA GCA CCA CTA TAC GGC CTA CTA|GCT ACA TGG CCA GTT AGA CGA CTA ACA GGC GCC ATA GTA ATT CGTACGGAACC
Pro Arg Gly Asp Met Pro Asp Asp Arg Cys Thr Gly Gln Ser Ala Asp Cys Pro Arg Tyr His OC*
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B.

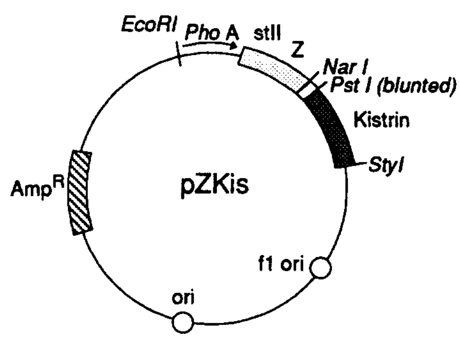

Fig. 2. Construction of a synthetic kistrin gene and the expression phagemid, pZkis. (**A**) Unique restriction sites are shown; junctions between synthetic DNA fragments are indicated by "|". The linker to connect *NarI* to the blunted *PstI* is boxed and contains the sequence Ala-Pro-Gly-*Phe-Ala-His-Tyr*-Gly-Lys, where Ala-Pro is the end of the Z domain, Phe-Ala-His-Tyr is the recognition site for the pentamutant subtilisin which cleaves at the carboxy terminal side of Tyr, and Gly-Lys is the beginning of kistrin. (**B**) The phagemid pZkis contains the alkaline phosphatase promoter (*phoA*), stII signal sequence, one Z domain of *S. aureus* protein A, the linker and synthetic kistrin gene described above, an ampicillin resistance gene, and pMB1 and F1 origins of replication (see Materials and Methods section).

performed essentially as described by Carter.[19] Clones were then analyzed by dideoxy sequence analysis[17] for the sequence encoding the desired change. The plasmid was then transformed into *E. coli* strain W3110 *ton*A (ATCC 27325) for expression.

Purification

Overnight cultures of W3110 *ton*A pZKis were grown in 2.5 mL 2YT media[20] containing 50 μg/ml carbenicillin and used to inoculate a 1-liter flask containing 250 ml of 2YT, 50 μg/ml carbenicillin. Cells were grown for 16 hr at 37°C, harvested by centrifugation (10,000*g*, 10 min), and the cell pellets frozen at −20°C for 1 hr. The frozen pellets were suspended in 12.5 ml of 10 mM Tris-HCl, pH 7.5 and shaken at 4°C for 1 hr to release the periplasmic contents. Cell debris was removed by centrifugation (10,000*g*, 10 min) and the supernatant decanted into a tube containing 0.25 ml of IgG Sepharose (Pharmacia) equilibrated in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween 20. The tube was shaken at room temperature for 20 min; the IgG Sepharose was allowed to settle and the supernatant was discarded. The resin was washed twice with 10 ml of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween 20, and once with 10 ml of 0.5 mM ammonium acetate before being poured into a column. Z-Kistrin fusion proteins were eluted with 1.5 ml of 1 N acetic acid, lyophilized, resuspended in 50 μl of 100 mM Tris-HCl, pH 8.6, 0.05% Tween 20, and digested with the pentamutant subtilisin (10% w/w) for 48 hr at 37°C. Kistrin mutants were purified from the Z domain and the pentamutant subtilisin by reverse-phase HPLC (C_{18}) with a gradient of 10–40% (v/v) acetonitrile in 0.1% (v/v) TFA[1] and were

stored at 4°C; kistrin is stable in 20% (v/v) acetonitrile, 0.1% (v/v) TFA.

FAB-Mass Spectrometry

Purified kistrin mutants (ca. 200 pmol each) were dried on a Savant speed vac concentrator and analyzed by fast atom bombardment-mass spectrometry (FAB-MS) to verify the expected atomic mass as described previously.[1]

Amino Acid Analysis

Kistrin mutants were dried as above and quantitated by amino acid analysis as reported by Dennis et al.[1] Recombinant kistrin was also sequenced as previously described.[1]

Kistrin/GP IIb-IIIa ELISA and Platelet Aggregation Assays

For the kistrin/GP IIb-IIIa solid phase ELISA, microtiter plates were coated with kistrin (2 μg/ml overnight at 4°C) and blocked with TACTS buffer (20 mM Tris-HCl pH 7.5/0.02% NaN_3/2 mM $CaCl_2$/0.05% Tween 20/150 mM NaCl) containing 0.5% bovine serum albumin for 1 hr. After washing with phosphate-buffered saline containing 0.01% Tween 20 (PBST), samples to be tested were added followed by purified GP IIb-IIIa (5 μg/ml) in TACTS, 0.5% bovine serum albumin. After a 1 hr incubation, the plate was washed (PBST) and monoclonal AP3 (1 μg/ml) was added. After another 1 hr incubation and PBST wash, goat anti-mouse IgG conjugated to horseradish peroxidase (Tago) was added. After a final PBST wash the plate was developed by adding 0.67 mg *o*-phenylenediamine dihydrochloride per ml of 0.012% H_2O_2, 22 mM sodium citrate, 50 mM phosphate, pH 5.0. The reaction was stopped with 2 M H_2SO_4 and A_{492} was recorded. A four parameter fit[21] was used to estimate the half maximal inhibition concentration (IC_{50}). The reported kistrin/GP IIb-IIIa IC_{50} values are the average of at least two separate determinations. Platelet aggregation assays were performed in human platelet-rich plasma as previously reported.[1] These IC_{50} values are the average of two separate determinations except for P48A, R56A, R66A, and P68A which were measured once.

RESULTS

Expression, Purification, and Cleavage of Z-Kistrin Fusion Proteins

Kistrin was expressed as a Z-domain fusion protein under the control of the alkaline phosphatase promoter in *E. coli*. A synthetic gene encoding kistrin and a proteolysis site for the pentamutant S24C/H64A/E156S/G169A/Y217L subtilisin BPN'[15] was constructed as shown in Figure 2. Although 2-fold higher expression was attained when cells were grown in a low phosphate media compared to 2YT, more fusion protein was obtained per liter using 2YT due to the higher cell density obtained (10-fold). All of the kistrin mutants were constructed in pZkis and expressed and purified as Z-kistrin fusion proteins as described in Materials and Methods. The fusion proteins were directed to the periplasm of *E. coli* by virtue of the stII signal sequence. Kistrin and mutants were released from the periplasmic space by osmotic shock and affinity purified on IgG Sepharose. Z-Kistrin fusions were quantitatively (>90% yield based on HPLC peak height) and specifically cleaved by the pentamutant subtilisin. Kistrin mutants were then purified to homogeneity by reverse-phase HPLC (Fig. 3); only minor changes were observed in retention times for the mutants (± 2% acetronitrile). The yield of kistrin mutants was generally about 150 to 300 μg/l of culture, although for some mutants (D15A, L21A, Q26A, T58A, P65A, H68A); the yield was lower (about 20–40 μg/l). Two mutants, F38A and Q60A, were not expressed at detectable levels.

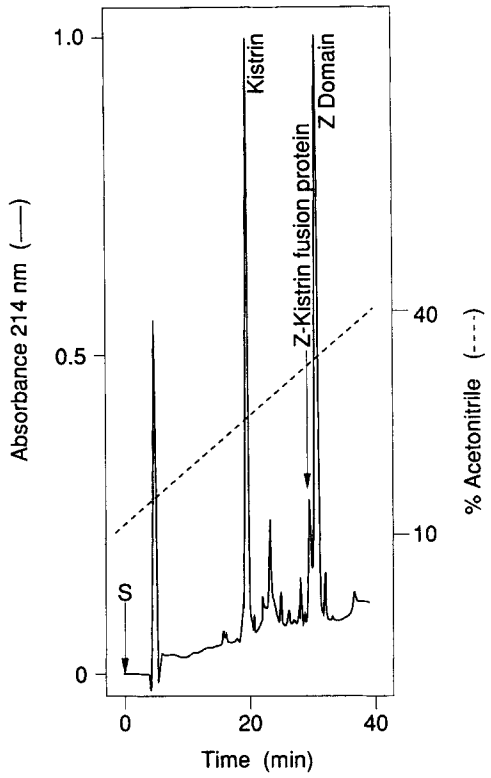

Fig. 3. Reverse-phase HPLC purification of recombinant kistrin. After cleavage with the pentamutant subtilisin, kistrin (ca. 100 μg) was purified from the Z domain and other products by C_{18} reverse-phase HPLC (10 to 40% CH_3CN/0.1% TFA in 40 min). Peaks corresponding to kistrin, Z-kistrin fusion, and the Z domain are labeled. Similar profiles were observed for each of the kistrin mutants.

Characterization of Kistrin Mutants

Alanine replacements were made for every amino acid throughout kistrin except for positions where a

TABLE I. Fab-Mass Spectrometry of Selected Kistrin Mutants

	Observed molecular mass* (amu)	Calculated molecular mass† (amu)
Kistrin	7318.0 ± 2	7318.3
r-Kistrin‡	7318.4 ± 2	7318.3
R46A	7235.8 ± 2	7233.2
I47A	7277.3 ± 2	7276.2
R49A	7233.5 ± 2	7233.2
G50A	7332.9 ± 2	7332.3
D51A	7274.6 ± 2	7274.3
P53A	7291.8 ± 2	7292.3
D54A	7274.3 ± 2	7274.3
D55A	7273.7 ± 2	7274.3
R66A	7234.1 ± 2	7233.2
H68A	7253.4 ± 2	7252.3

*Details of the analysis are reported in Materials and Methods. The data reported are corrected for the ionized M+1 (H^+) or M+23 (Na^+) peak that is observed.
†Molecular mass data were calculated from the sequence of the native proteins, assuming all cysteines form disulfide bonds.
‡r-Kistrin refers to recombinant kistrin.

glycine or alanine already existed (Fig. 1). Cysteine residues were also omitted from the scan to avoid disruption of the disulfide bonding pattern. Three additional mutants were made to the RGD region of kistrin: R49K, D51E, and a double mutant R49K/G50A. In addition to DNA sequence analysis, purified mutants were checked by FAB-MS and amino acid analysis. FAB-MS proved to be a rapid and sensitive method for verifying the correct change in molecular mass of the mutants (Table I). Amino acid analysis was used to quantitate the mutants and also provided an additional check on the altered composition (data not shown). In addition, amino terminal sequencing of recombinant kistrin verified that the correct sequence was intact and that no proteolysis had occurred.

The specific activities (IC_{50}) of kistrin and mutants were evaluated using both a kistrin/GP IIb-IIIa solid phase ELISA and a platelet aggregation inhibition assay. The kistrin/GP IIb-IIIa ELISA provided a direct comparison between kistrin mutants versus wildtype as measured by their ability to block binding of soluble GP IIb-IIIa to immobilized kistrin (Fig. 4); the IC_{50} value for wildtype kistrin (native or recombinant) is 10 ± 2 nM (n = 5). Mutants differing significantly from wild type were further characterized by their ability to inhibit platelet aggregation in human platelet rich plasma (Fig. 5); kistrin has an IC_{50} value in this assay of 132 ± 30 nM (n = 4). Data for mutants in both assays (Figs. 4 and 5) were normalized to native kistrin. The ratios of the IC_{50}(mutant)/IC_{50}(wild type) in the kistrin/GP IIb-IIIa ELISA for R49K, D51E, and R49K/G50A are 3.0 ± 0.3, >110, and 45 ± 0.1, respectively. In the platelet aggregation assay the ratios of the IC_{50}(mutant)/IC_{50}(wild type) are 1.8 ± 0.7, >100, and 3.6 ± 1.2, respectively.

DISCUSSION

The GP IIb-IIIa complex binds several different ligands including the adhesion proteins fibrinogen, fibronectin, von Willebrand factor, and vitronectin.[3,4] These ligands all contain the RGD sequence, a common recognition element in many adhesion proteins,[22,23] which is thought to play a critical role in binding to both GP IIb-IIIa as well as other integrin receptors. This is primarily based on evidence that RGD-containing peptides can (1) inhibit ligand binding to platelets,[24-27] (2) serve as affinity ligands to purify RGD-dependent integrin receptors,[28] (3) elicit antibodies that inhibit ligand binding to receptor,[29,30] and (4) directly bind to the receptor.[31,32]

Kistrin and other disintegrins[1,2] are considerably more potent GP IIb-IIIa receptor antagonists than linear RGD peptides.[24,33,34] This increased potency is presumably due to a favorable conformation in the RGD region of these proteins and/or the existence of additional binding determinants provided by the inhibitors. In this study, the interaction of kistrin with GP IIb-IIIa was examined by systematically replacing amino acids with alanine; a similar strategy has been used in studying other protein–protein interactions.[35-37] While having a minimal affect on secondary and tertiary conformations due to its small size and neutral character, alanine replacements eliminate side chain interactions beyond the β-carbon, allowing contributions made by individual side chains to be monitored.

The purification of microgram quantities of numerous kistrin mutants required a quick and efficient method relative to intracellular expression, CNBr digestion, purification, and refolding methodology employed making recombinant echistatin.[38] In this study kistrin is secreted, properly folded and easily purified. The stII signal sequence directed the Z-kistrin fusion protein to the periplasmic space of *E. coli*, free from intracellular proteases and the insolubility problems associated with inclusion bodies.[39] Purification, often the rate-limiting step in structure–function analysis of variant proteins, was simplified by utilizing the Z domain from protein A; mutant fusion proteins could be rapidly purified in a single column step using IgG Sepharose.[40]

Highly purified kistrin variants were readily recovered by HPLC following site-specific proteolysis of the linker region of the fusion protein with the pentamutant subtilisin.[15] Although commonly a problem in other cleavage strategies, no evidence for cleavage at internal sites was observed.[41] This reflects both the narrow substrate specificity of the pentamutant subtilisin and the fact that kistrin is highly resistant to proteolysis. All the disulfides appeared to form spontaneously and correctly as

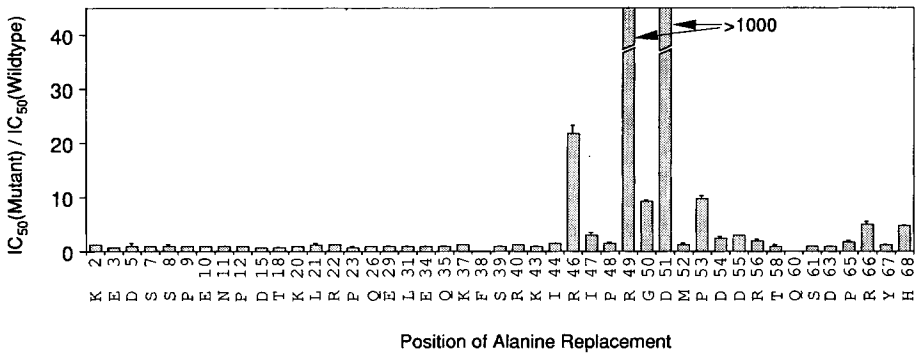

Fig. 4. The IC_{50} (mutant)/IC_{50} (wild type) ratios of alanine replacement mutants as measured by the kistrin/GP IIb-IIIa ELISA. The IC_{50} (mutant)/IC_{50} (wild type) ratio for each mutant as measured by the kistrin/GP IIb-IIIa ELISA is plotted above the sequence position and replaced amino acid. No value is shown for F38A and Q60A since no protein was obtained for these mutants. Alanine mutants were not made at the cysteine or glycine positions. Values were determined as described in Materials and Methods.

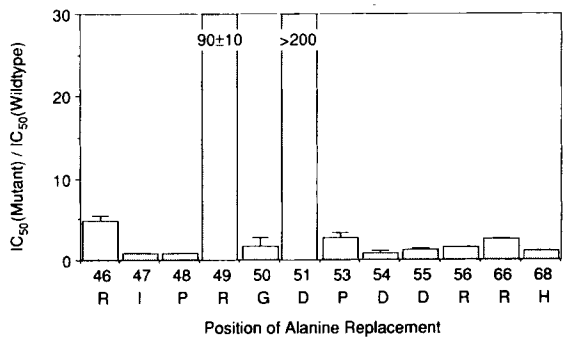

Fig. 5. The IC_{50} (mutant)/IC_{50} (wild type) ratios for alanine replacement mutants as measured by platelet aggregation. Values were determined as described in Materials and Methods.

judged by FAB-MS (Table I) and similarity of HPLC retention times; values for recombinant kistrin were within experimental error of native protein.

The kistrin/GP IIb-IIIa ELISA and platelet aggregation assays were used to monitor kistrin mutant interactions with GP IIb-IIIa. The kistrin/GP IIb-IIIa ELISA, which measures the ability of soluble kistrin or mutant to inhibit GP IIb-IIIa binding to immobilized kistrin, was used to measure the relative affinity of mutant versus wild type and served as a rapid screen for the alanine replacement mutants (Fig. 4). Inhibition of platelet aggregation, a more biologically relevant assay, was used to examine the ability of selected mutants to block fibrinogen from binding to the platelet receptor; in general a good correlation was observed for these assays (Figs. 4 and 5). Most of the alanine replacements in kistrin showed no difference in the kistrin/GP IIb-IIIa ELISA and served as a baseline to which mutants with reduced activity could be compared (Fig. 4). Also the fact that most of the kistrin mutants are as active as native kistrin provides additional evidence that the recombinant proteins are able to fold correctly.

Both R49 and D51 were found to be critical for the potency of kistrin as measured by both assays. In the kistrin/GP IIb-IIIa ELISA both R49A and D51A were >1,000-fold less potent than wildtype (Fig. 4); in the platelet aggregation assay, R49A and D51A are 90-fold and >200-fold less potent than wildtype, respectively (Fig. 5). The relative importance of these two residues is further supported by the conservative changes R49K and D51E. Mutant R49K shows a 1.8- and 3-fold decrease in activity compared to wild type in the platelet aggregation assay and kistrin/GP IIb-IIIa ELISA, respectively, while D51E shows a greater than 100-fold decrease in potency for these assays. This suggests that D51 is the most critical residue for the interaction of kistrin with GP IIb-IIIa.* The same relative importance of these two positions has been observed in peptides as well.[25,27,33]

Changes at the arginine position of the RGD sequence in other disintegrins have been examined previously; modifications of the corresponding glycine or aspartate have not been explored. In echistatin replacement of arginine with alanine (R24A) leads to a 19-fold decrease in potency in platelet aggregation compared to wild type.[9] Barbourin, which has a KGD sequence instead of the RGD sequence found in all other known venom GP IIb-IIIa antagonists, is 2.4-fold less potent than kistrin as a platelet aggregation inhibitor.[42] Finally, the corresponding arginine in eristocophin has been replaced with lysine (R29K) which leads to a 3-fold less potent platelet aggregation inhibitor than wild type. The observed loss in potency for kistrin mutants R49A (90-fold) and R49K (1.8-fold) is in general agreement with these results.

*Data from a solid phase Fg/GP IIb-IIIa ELISA[1] shows that D51A was ca. 1000-fold less potent than R49A in this assay.

Comparison of the peptides GRGDSP and GRADSP (IC_{50} = 35 and 800 μM, respectively) demonstrates that in linear peptides, the glycine of the RGD is important for the inhibition of fibrinogen binding to platelets.[27] However, the corresponding change in kistrin, G50A, is only 2.5-fold less potent as a platelet aggregation inhibitor than wild type. A glycine in this position may be required to provide the peptides with favorable main chain torsion angles. Some of the distorsion caused by alanine in the G50A mutant can apparently be compensated in kistrin, whose structure is conformationally restrained by 6 disulfides. The double mutant R49K/G50A is still a very potent GP IIb-IIIa antagonist and platelet aggregation inhibitor. We conclude that while the aspartate of the RGD sequence appears to be required for high affinity binding to GP IIb-IIIa, at least in the context of kistrin and related proteins, the corresponding arginine and glycine are not.

Alanine replacements of residues flanking the RGD sequence displayed altered IC_{50} values in the kistrin/GP IIb-IIIa ELISA (R46A, I47A, P53A, D54A, D55A, R56A, R66A, and H68A); however, only R46A, P53A, and R66A displayed significantly different potency in platelet aggregation. This may be due to the difference in binding of purified GP IIb-IIIa versus platelet associated GP IIb-IIIa or the difference in binding affinities of kistrin ($K_{d\ kistrin}$ = 1.7 ± 0.2 nM) versus fibrinogen ($K_{d\ Fg} \approx 100$ nM) to GP IIb-IIIa in ADP-activated human washed platelets,[1] leading to sensitivity differences in the assays.

Kistrin mutants P53A and R66A are less potent inhibitors of platelet aggregation compared to wild type by 2.5- to 3-fold. While P53 might be suspected as being important for the structure of kistrin, proline is not conserved in this position and is absent from most of the known snake venom inhibitor sequences (Fig. 1). Carboxy terminal deletions have suggested a role for the C terminus of echistatin[9] which lends support to a possible role for R66. This arginine is highly conserved except in the genus *Bitis* where it is a tryptophan.

In all the published snake venom sequences, the residue corresponding to R46 is an arginine except in echistatin where it is a lysine. Mutant R46A showed the largest effect on inhibition of platelet aggregation outside the RGD sequence, suggesting that a positive charge in this area may be beneficial for binding to GP IIb-IIIa. The 5-fold decrease in inhibition of platelet aggregation is still minor compared to the magnitude of the decreases observed for R49A and D51A. Most of the binding energy is provided by R49 and D51 with little attributed to a second site. Therefore the high potency seen in kistrin and other snake venom proteins is likely due to proper presentation of the RGD conformation as opposed to additional contacts provided by regions outside the RGD region. Significant improvement in binding affinity to GP IIb-IIIa has been observed when small RGD peptides have been cyclized to reduce conformational flexibility thus reducing an entropic barrier to binding.[43–45,45] These observations lend further support to this conclusion.

The importance of a favorable conformation of the RGD sequence is also supported by data from other potent RGD-containing protein GP IIb-IIIa antagonists recently isolated. Decorsin and ornatin are present in leeches and are similar to the *Viperidae* venom proteins only in the RGD region of these proteins.[47,48] Mambin, a protein from the venom of an Elapidae snake, *Dendroaspis jamesonii*, is also a potent inhibitor of platelet aggregation, presumably solely by virtue of its presentation of the RGD sequence. Based on sequence homology and molecular modeling, mambin is structurally related to the short chain neurotoxins except for an RGD sequence present in an extended loop somewhat similar to that observed in kistrin.[49]

Mutagenesis of kistrin was initiated without the detailed knowledge of the protein structure. Recently the kistrin structure has been solved by 2-D NMR and shown to be a series of tightly packed loops, lacking secondary structure, held together by the 6 disulfide bonds (Fig. 6).[11] The RGD sequence lies at the apex of a long extended loop across the surface of the protein; the loop consists of two antiparallel strands and displays the RGD away from the protein core. Data from the alanine scan reveal that most of the mutations are silent, consistent with the proposed structure and a primary role for the RGD region. Interestingly, the loop containing the RGD sequence is conformationally less restricted than the rest of the protein. Similar results have been observed for the 2-D NMR derived structure of echistatin.[50] Although the RGD-containing loop is the most flexible region of kistrin, the conformation of the RGD region is still considerably more restricted than that of a linear RGD peptide. This decrease in conformational entropy of free kistrin compared to a linear peptide is consistent with the conclusions from the alanine scan where conformational constraint of the RGD region alone would seem to be important for high affinity binding.

The homology among the disintegrin snake venom platelet aggregation inhibitors is striking, especially in the amino terminal half of the proteins (Fig. 1).[1,2,42] The results of the alanine scan of kistrin cannot explain this high degree of conservation, since only a few residues were found to be important for GP IIb-IIIa antagonist activity. The amino terminal region of kistrin is distant from the exposed RGD loop and is not involved in direct binding to GP IIb-IIIa; echistatin lacks this sequence entirely. However, this region is very highly conserved in all other disintegrins and may be related to protein stability or perhaps some as yet unidentified function.

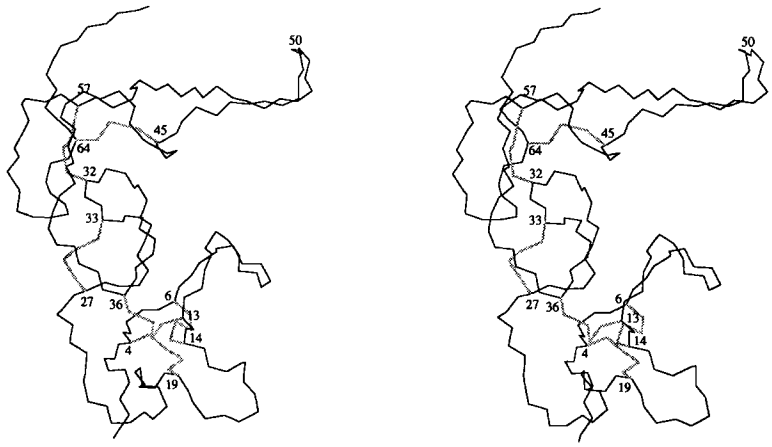

Fig. 6. Stereoview of the 2D NMR solution structure of kistrin containing the lowest violation of the constraints; the backbone atoms are displayed.[11] The side chain atoms of the 12 cysteines have been drawn in gray and the Cα's have been labeled. The Cα of Gly-50 has also been labeled.

The true biological role of these proteins in the venom remains unknown.

Recently, disintegrin domains have been observed within other proteins, notably the β subunit of PH-30, a heterodimeric guinea pig sperm surface protein involved in sperm-egg fusion,[51] and HR1B, a hemorrhagic protein from the venom of the viper *Trimeresurus flavoviridis*.[52] The overall similarity of these proteins to the disintegrins is about 50%; all of the cysteine residues are conserved. However, in PH-30 β and HR1B, the sequences TDE and ESE are present, respectively, at the position corresponding to the RGD sequence found in the disintegrins. Data from the alanine scan of kistrin, coupled with its tertiary structure determined by 2D NMR,[11] lead one to speculate that the loop which contains RGD in kistrin may also play an important functional role in these proteins. Furthermore, HR1B contains a metalloproteinase domain at its amino terminus. Translation of the entire cDNA sequence for kistrin reveals that it also contains an amino-terminal metalloproteinase domain.[53] The recurrence of metalloproteinase and disintegrin domains in the same protein suggests a synergistic function. In addition to their ability to inhibit cell–cell and cell–matrix interactions, another role of the disintegrins, which are somewhat resistant to proteases, may be to target the metalloproteinase to its site of action.

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