Polymerization-Defective Fibrinogen Variant γD364A Binds Knob "A" Peptide Mimic[†,‡]

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ABSTRACT: Fibrin polymerization is supported in part by interactions called "A:a". Crystallographic studies revealed γ364Asp is part of hole "a" that interacts with knob "A" peptide mimic, GPRP. Biochemical studies have shown γ364Asp is critical to polymerization, as polymerization of variants γD364A, γD364H, and γD364V is exceptionally impaired. To understand the molecular basis for the aberrant function, we solved the crystal structure of fragment D from γD364A. Surprisingly, the structure (rfD-γD364A+GP) showed near normal "A:a" interactions with GPRP bound to hole "a" and no change in the overall structure of γD364A. Of note, inspection of the structure showed negative electrostatic potential inside hole "a" was diminished by this substitution. We examined GPRP binding to the γ364Asp variants in solution by plasmin protection assay. We found no protection of either γD364H or γD364V but partial protection of γD364A, indicating the peptide does not bind to either γD364H or γD364V and binds more weakly than normal to γD364A. We also examined protection by calcium and found all variants were indistinguishable from normal, suggesting the global structures of the variants are not markedly different from normal. Our data imply that γ364Asp per se is not required for knob "A" binding to hole "a"; rather, this residue's negative charge has a critical role in the electrostatic interactions that facilitate the important first step in fibrin polymerization.

Fibrinogen is a plasma glycoprotein consisting of a dimer of three polypeptide chains, Aα, Bβ, and γ. The molecule is folded such that the N-termini of all six chains originate at the center of the molecule, called the E nodule. The C-termini of the chains extend from the center as two pairs of three chains. The C-termini of the Bβ and the γ chains fold into independent globular regions that are located at the peripheral ends of the molecule forming two identical D nodules. The E and D regions are linked by α-helical coiled-coil connectors forming a trinodular structure (*1*). Laser tweezers-based force spectroscopy data suggest that the central E region in fibrinogen forms intramolecular interactions with the two Aα carboxyl termini (αC domains) (*2*).

The conversion of fibrinogen to fibrin monomer is catalyzed by the enzyme thrombin, which cleaves two pairs of short fibrinopeptides from the N-termini of the Aα (called FpA)[1] and Bβ (called FpB) chains, exposing sites designated as knobs "A" and "B", respectively (*3*). The exposed knob "A" binds to a complementary binding site in the γ-chain called hole "a" forming "A:a" interactions (*3, 4*). These interactions lead to a double-stranded fibrin protofibril with a half-staggered overlap between molecules in different strands (*5*). Cleavage of FpB occurs primarily during protofibril growth, leading to the binding of knob "B" to a complementary binding site in the β-chain called hole "b" forming "B:b" interactions (*6*). This event enhances the rate and extent of lateral aggregation of protofibrils and leads to the formation of thicker fibers (*7*).

The location of the binding holes and possible models for knob-hole interactions are known from X-ray crystallographic studies using two synthetic peptide analogues of knobs "A" and "B". When fibrinogen fragment D and double-D are crystallized in the presence of both peptide analogues, the knob "A" peptide mimic, GPRP, forms H-bond interactions

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[‡] The atomic coordinates have been deposited in the Protein Data Bank (www.rcsb.org) under the access code 3BVH (rfD-γD364A+GP).

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[1] Abbreviations: rfD, recombinant fibrinogen fragment D; rfD+BOTH, structure of normal recombinant fibrinogen fragment D with both GPRP and GHRP bound; rfD-γD364A+GP, structure of fragment D from recombinant γD364A fibrinogen with GPRP bound; GPRP, Gly-Pro-Arg-Pro-amide; GHRP, Gly-His-Arg-Pro-amide; FpA, fibrinopeptide A; FpB, fibrinopeptide B; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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with residues γ364Asp, γ330Asp, γ329Gln, and γ340His found in hole "a". In the same manner, the knob "B" peptide mimic, GHRP, interacts with residues Bβ397Glu, Bβ398Asp, and Bβ432Asp in hole "b" (*8, 9*). High-resolution structural data also showed that the γ- and β-modules, containing the binding holes, are highly homologous (*8, 9*).

The significance of the interactions between GPRP and residue γ364Asp is known from studies of two previously identified dysfibrinogens with substitutions at γ364. The substitution of histidine for γ364Asp (Fibrinogen Matsumoto I) was found in an individual who is heterozygous for the mutation (*10*). The patient showed prolonged clotting time, although no overt coagulation defect, neither bleeding nor thrombosis. Another individual, who presented with numerous episodes of deep venous thrombosis, was found to be heterozygous for the valine mutation, γD364V (Fibrinogen Melun) (*11*). Previous works have used recombinant variant fibrinogens (*12, 13*) to show that the residue γ364Asp is critical to normal thrombin-catalyzed polymerization. Nevertheless, the molecular basis for the polymerization impairment in these γ364Asp variants remains unclear.

In the current study, we examined the molecular nature of "A:a" interactions in γ364Asp variants. We crystallized fragment D from γD364A in the presence of the synthetic peptides GPRP and GHRP and solved the 2.6 Å structure, which we called rfD-γD364A+GP as the crystals contained only GPRP. We used proteolytic fragmentation to study binding of GPRP in γD364A, γD364H, and γD364V. Our data suggest that "A:a" interactions are weak in γD364A and absent in both γD364H and γD364V. While our structural data showed that the alanine substitution in γD364A does not alter hole "a" and this variant still binds knob "A", biochemical data suggest that the loss of γ364Asp weakens "A:a" interactions and diminishes electrostatic steering that may be critical for normal fibrin polymerization.

EXPERIMENTAL PROCEDURES

Reagents. All chemicals were of reagent grade and were purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise. The peptides GPRP and GHRP were synthesized by the Protein Chemistry Laboratory at the University of North Carolina at Chapel Hill (Chapel Hill, NC). Cell culture media with normal recombinant fibrinogen were obtained from the National Cell Culture Center (Minneapolis, MN). Cyanogen bromide-activated Sepharose 4B and Superose 6 resins were purchased from Amersham Biosciences (Piscataway, NJ). Monoclonal IF-1 antibody was purchased from Kamiya Biomedical (Seattle, WA).

Expression and Purification of Recombinant Fibrinogen γD364A, γD364H, and γD364V. Normal and variant recombinant fibrinogens γD364A, γD364H, and γD364V were synthesized in CHO cells as described previously (*12, 13*). Large-scale protein expression was carried out in serum-free medium in roller bottles. Media containing secreted fibrinogen were harvested periodically. After addition of protease inhibitors, the media were stored at −20 °C (*14*).

Recombinant fibrinogen was purified as described (*15*). Briefly, fibrinogen was precipitated from the media with ammonium sulfate in the presence of a cocktail of protease inhibitors. The precipitate was resuspended in buffer containing 10 mM $CaCl_2$ and applied to a Sepharose 4B column coupled with the fibrinogen specific monoclonal antibody, IF-1. Fibrinogen was eluted from the column with buffer containing 5 mM EDTA, dialyzed against HBS (20 mM HEPES, pH 7.4, 150 mM NaCl) and 1 mM $CaCl_2$ for one exchange and then extensively dialyzed against HBS, and stored at −80 °C. The integrity of the polypeptide chains and purity of the recombinant protein were analyzed by SDS−PAGE under reduced and nonreduced condition following the method of Laemmli (*16*).

Preparation and Purification of Fragment D. Fragment D was prepared from γD364A fibrinogen by controlled trypsin digestion, according to the method used for normal recombinant fibrinogen (*9*). Briefly, $CaCl_2$ was added to 9 mg of γD364A (final concentration of 1.8 mg/mL) in HBS to a final concentration of 20 mM. Digestion was initiated by adding 50 μL of immobilized TPCK trypsin (Pierce Biotechnology Inc., Rockford, IL). The reaction was allowed to proceed over a period of days until SDS−PAGE revealed bands that corresponded to the molecular weights of fragments D and E. Digestion was stopped by removing the trypsin beads with centrifugation at 3000 rpm (Sorvall RC-3) for 10 min.

Fragment D was purified from the γD364A digest by lectin affinity chromatography based on the previously described method (*17, 18*). Briefly, the digest was loaded onto a concanavalin A−Sepharose column equilibrated with 10 mM Tris (pH 7.4) and 1 M NaCl (loading buffer). Fragment E was retained on the column while fragment D was eluted. The column was washed extensively with the loading buffer until all fragment D was collected in the eluate. Purified fragment D from γD364A was dialyzed against 50 mM Tris (pH 7.4), concentrated a 100-fold with a centrifugal filter device (50 kDa MW cutoff, Millipore Corp., Billerica, MA), and stored at 4 °C.

Crystallization of Fragment D from γD364A. Purified γD364A fragment D at 6.8 mg/mL was cocrystallized with a mixture of 20 mM GPRP and 4 mM GHRP by sitting-drop diffusion at 4 °C as described (*9, 19*). These peptide concentrations were tested after attempts at crystallizing with 4 mM of both peptides failed. Crystals were grown from drops containing 3 μL of fragment D/peptide in 50 mM Tris-HCl, pH 7.4, mixed with an equal volume of crystallant solution in the well which contained 50 mM Tris-HCl, pH 8.5, 2 mM NaN_3, 12.5 mM $CaCl_2$, and 9% (w/v) PEG 3350. Single crystals appeared within days following streak seeding with crystals of normal recombinant fragment D (rfD) without added peptides.

X-ray Diffraction Data Collection and Structure Determination. Data collection for crystals of fragment D from recombinant γD364A fibrinogen (rfD-γD364A+GP) was carried out at 100 K using beamline X29 at the National Synchrotron Light Source at Brookhaven National Laboratory. A single crystal was selected and soaked in the same crystallant solution with 20% (v/v) glycerol, 20 mM GPRP, and 4 mM GHRP. The crystal was then looped and flash-frozen in liquid nitrogen. Diffraction data were processed with DENZO and ScalePack (*20*).

The structure of the variant was solved by rigid body minimization followed by simulated annealing using the rfD structure without peptides bound (PDB code 1LT9) as a starting model. The resulting model was refined using CNS (*21*), and the model was improved by manual fitting in the

Table 1: Crystallographic Data and Refinement Statistics

Data Statistics	
resolution (Å)	50.0−2.6
space group	$P2_12_12_1$
cell constants (Å)	$a = 89.8$, $b = 94.9$, $c = 225.7$
molecules/asymmetric unit	2
total observations	257228
unique reflections	66878
mean redundancy	3.8
R_{sym}[a] (%) (highest shell)	11.8 (31.1)
completeness (%) (highest shell)	82.4 (94.2)
mean I/σ (highest shell)	13.7 (4.8)
Refinement Statistics	
R_{cryst}[b] (%)	22.1
R_{free}[c] (%)	26.4
average B factor	37.3
no. of model atoms	10618
no. of solvent sites	446
RMS Deviations from Ideals	
bond length (Å)	0.006
bond angle (deg)	1.24

[a] $R_{sym} = \sum|I - \langle I\rangle|$, where I is the observed intensity and $\langle I\rangle$ is the average intensity of multiple symmetry-related observations of that reflection. [b] $R_{cryst} = \sum(|F_o| - |F_c|)/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. [c] R_{free} is the R-factor based on data withheld from structural refinement.

program O (*22*) using sigmaA-weighted $|2F_o - F_c|$ and $|F_o - F_c|$ electron density maps (*23*). Several cycles of least-squares minimization, simulated annealing, and individual temperature factor refinement applying weak noncrystallographic restraints in the first cycles were done before water molecules were added to complete the model. Furthermore, *cis*-peptide bonds were added at positions Bβ407 and γ339. After all these refinement steps, the R_{cryst} and R_{free} converged to the values reported in Table 1.

Plasmin Protection Assay. Normal recombinant or variant fibrinogens (0.25 mg/mL) in HBS buffer containing 5 mM $CaCl_2$, 5 mM EDTA, and 4 or 20 mM GPRP (supplemented with 5 mM EDTA) were incubated with 4.8 μg/mL plasmin (Haematologic Technologies, Inc., Essex Junction, VT) for 4 h at 37 °C. The reactions were stopped by adding SDS−PAGE loading dye and heating at 100 °C for 5 min. The plasmin digests were then analyzed on 7.5% gels that were stained with Coomassie brilliant blue.

RESULTS

Structure of rfD-γD364A+GP. We obtained high quality diffraction data with the γD364A fragment D crystallized in the presence of both GPRP and GHRP and were able to solve the structure at 2.6 Å. Because only GPRP was found in the crystal structure, we called this rfD-γD364A+GP.

The space group and unit cell dimensions of rfD-γD364A+GP were isomorphous to those of normal rfD in the presence and absence of peptides; thus we solved the structure of the variant by rigid body minimization followed by simulated annealing using the rfD structure (PDB code 1LT9) as a starting model. A total of 653 amino acid residues in each molecule of the asymmetric unit (α129−190, β161−458, and γ102−394) were elucidated from the electron density. The substitution of the Asp to Ala at γ364 was evident. Structure alignment of the normal and γD364A fragment D showed excellent agreement with an RMSD of 0.45 Å over the aligned Cα-atomic positions, showing that the global structure of rfD-γD364A was not changed by the alanine substitution. Moreover, we found two calcium ions bound in the γ1 and β1 sites, as was observed previously for rfD-BOTH (*9*).

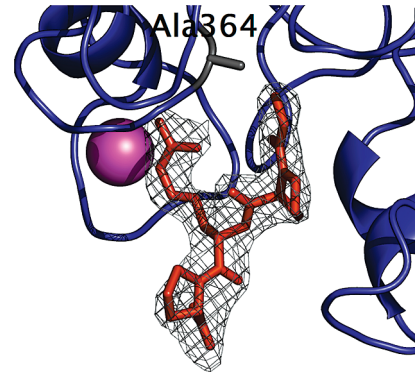

FIGURE 1: GPRP was bound to hole "a" in the γ-chain. The $|F_o - F_c|$ electron density around the peptide (brown) was contoured at 3.0σ and is shown as gray mesh. The γ1 calcium-binding site located near hole "a" is shown as a magenta sphere.

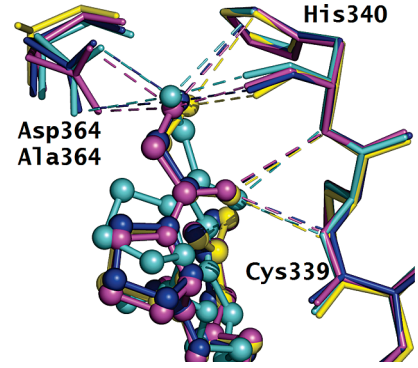

FIGURE 2: Interaction of the N-terminus of GPRP with hole "a" seen in various crystal structures. The peptide in each structure is shown in balls and sticks. The structure in cyan is obtained from normal recombinant fragment D with peptides bound, rfD+BOTH (1LTJ). The structure in blue corresponds to the cross-linked DD fragment with peptides bound (1FZC) while the structure in magenta is from the C-terminal 30-kDa γ-chain fragment with GPRP (2FIB). The rfD-γD364A+GP (3BVH) structure is shown in yellow. In rfD-γD364A+GP structure, the ionic interaction between γ364Asp and the free amino group of GPRP is lost.

Peptide Binding in rfD-γD364A+GP. A surprising feature of the rfD-γD364A+GP structure was the presence of GPRP in hole "a" (Figure 1). We had anticipated that the loss of the ionic interaction between the aspartate side chain and the free amino group of GPRP would deter peptide binding. Nevertheless, the peptide in hole "a" of the γ-chain was evident in the calculated $|2F_o - F_c|$ and $|F_o - F_c|$ electron density maps.

We compared the interactions of the N-terminal glycine of GPRP in rfD-γD364A+GP to other known structures with the peptide bound to hole "a" (*8, 9, 24*). In all of the structures shown in Figure 2, the carboxyl backbone of glycine forms H-bonds with the nitrogen backbones of γ340His and γ339Cys while the free amino group interacts with the nitrogen side chain and carboxyl backbone of γ340His. Additionally, the positively charged free amino group of GPRP forms ionic interaction with the negatively charged carboxylate side chain of γ364Asp. This ionic interaction is lost in γD364A.

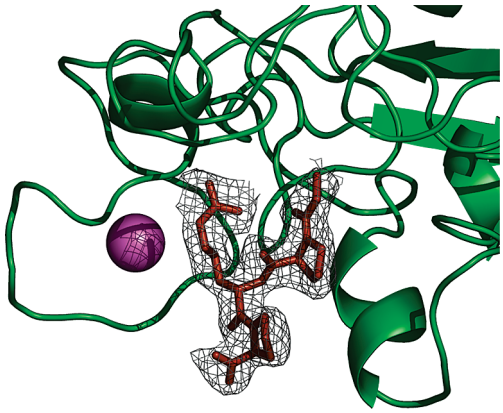

FIGURE 3: GPRP was bound to hole "b" in the β-chain. In the presence of 4 mM GHRP and 20 mM GPRP, the latter was bound to hole "b". The $|F_o - F_c|$ electron density around GPRP (brown) was contoured at 3.0σ and is shown as gray mesh. The β1 calcium-binding site located near hole "b" is shown as a magenta sphere.

It is also notable that GPRP, and not the knob "B" peptide mimic GHRP, was bound to hole "b" in the β-chain. This was evident from the presence of a negative difference map around the histidine side chain (data not shown) when GHRP was modeled in hole "b"; in contrast, GPRP modeled at this site fit well with the electron density map (Figure 3). We conclude that 20 mM GPRP was sufficient to prevent binding of GHRP at 4 mM to fragment D. This noncanonical "A:b" interaction was also seen in the structure of recombinant fibrinogen variant γD298,301A fragment D (rfD-γD298, 301A+GP) when crystallized in the presence of only GPRP (*17*). This implies that "A:b" interactions are possible during fibrin polymerization but the circumstances at which they occur and their significance are unknown.

Impact of the γD364A Substitution to Hole "a". Gross visual comparison of the normal rfD+BOTH and rfD-γD364A+GP structures (Figure 4A) revealed a roomier hole "a" in γD364A than in normal fibrinogen. In a detailed comparison, we found no differences in the position or orientation of any residue within 4.5 Å of the peptide (Figure 4B). Moreover, the alanine substitution did not alter the positions of residues γ338Lys-γ339Cys-γ340His, a triad of polar interactions that contribute to the stability of GPRP binding (Figure 4C). Indeed, we found a *cis*-peptide bond between γ338Lys-γ339Cys, as has been seen in the structure of a 30 kDa C-terminal fragment of γ-chain (*24*), all other fragment D and cross-linked double-D structures (*8, 9, 17, 25, 26*). As is true for many protein structures (*27*), this *cis*-peptide bond in fibrinogen is located in a functionally important region of the molecule. Here it allows the γ339Cys backbone nitrogen to interact with the carbonyl group of glycine in GPRP. We also examined the position of γ363Tyr. Doolittle et al. recently noted a conformational change in γ363Tyr-γ364Asp associated with GPRP binding (*28*). Specifically, when hole "a" is occupied, γ364Asp shifts slightly to interact with the peptide while γ363Tyr moves ~3 Å toward the arginine of GPRP forming favorable amino−aromatic interactions (*29*). In the γD364A structure, there was no shift in the alanine side chain. Nevertheless, γ363Tyr in the γD364A structure was positioned as it is in the peptide bound structure of the normal protein, rfD-BOTH, forming amino−aromatic interactions with the peptide arginine.

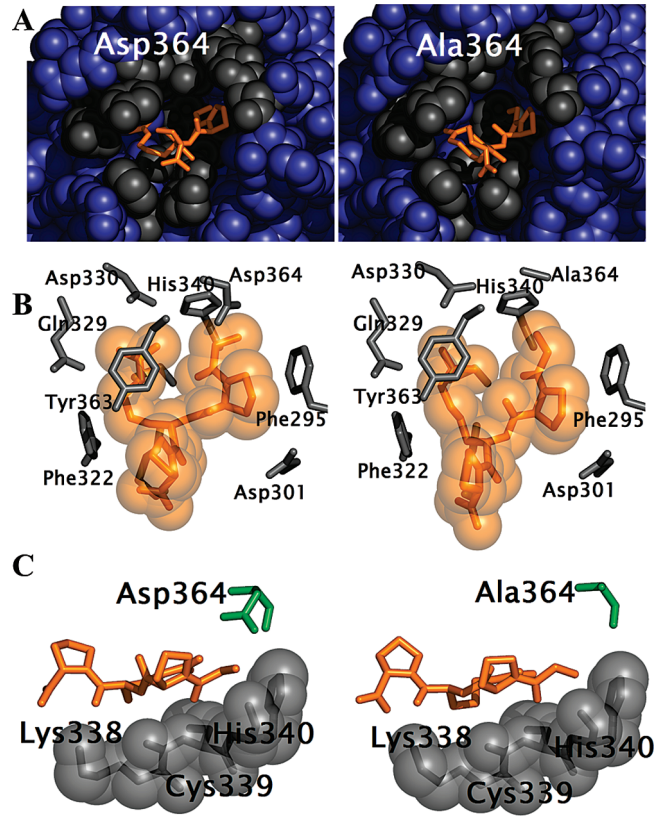

FIGURE 4: Comparison of hole "a" in rfD+BOTH and rfD-γD364A+GP. Panel A: GPRP bound to hole "a" is shown in orange. Gross inspection shows that γD364A makes a roomier hole "a". Panel B: The set of residues forming polar and hydrophobic contacts with the GPRP is the same for normal and γD364A fragment D. Note that the alanine substitution did not introduce significant changes within residues in the hole. Panel C: The highly conserved γ338Lys-γ339Cys-γ340His triad that supports peptide binding is not altered in γD364A.

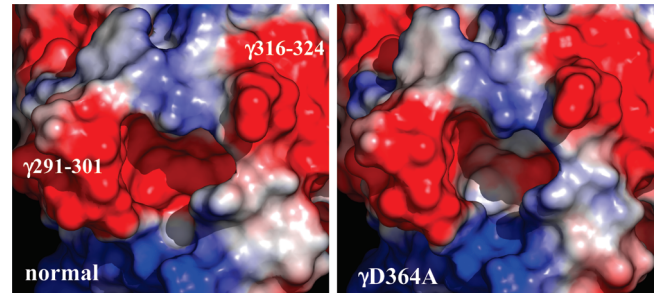

FIGURE 5: Charge distribution comparison of hole "a" in normal and γD364A. The structures show patches of surface-accessible negative electrostatic potential (shown in red) that surround hole "a". These areas consist of loops γ316−324 and γ294−301 that contain calcium binding sites. Inside the cavity of hole "a" are residues γ364Asp and γ330Asp that contribute to the local negative charge. Note the diminished negative charge in hole "a" of γD364A.

Our data suggest that the conformational change in γ363Tyr, which is seen in the presence of peptide, is independent of γ364Asp.

We also compared the charge distribution in hole "a" of γD364A and rfD-BOTH (Figure 5). As noted from the earlier crystal structure of the C-terminal 30 kDa γ-chain fragment (*30*), loops surrounding hole "a" region provide large areas of negatively charged surface. Loop γ316−324, for instance, provides a surface accessible negative electrostatic potential.

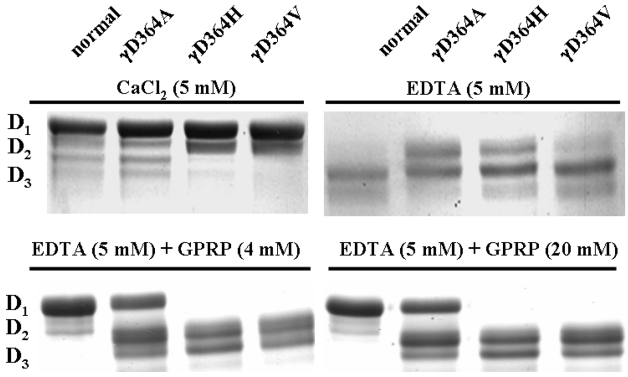

FIGURE 6: Plasmin protection assay. Normal recombinant or variant fibrinogens (0.25 mg/mL) in HBS buffer containing 5 mM $CaCl_2$, 5 mM EDTA, and 4 or 20 mM GPRP (supplemented with 5 mM EDTA) were incubated with 4.8 μg/mL plasmin for 4 h at 37 °C, and the digests were analyzed on 7.5% SDS−PAGE. The fibrinogen degradation products are indicated on the left.

This loop contains γ318Asp and γ320Asp that binds calcium (*8, 9, 30*). Alanine substitutions for these carboxylate moieties have been shown to impair calcium binding, fibrin polymerization, and fibrinogen-mediated platelet aggregation (*31*). Another negatively charged surface adjacent to hole "a" is loop γ294−301 which contains γ298Asp and γ301Asp that also bind calcium. Studies of recombinant fibrinogen γD298,301A suggested that changes in loop γ294−301 could possibly alter the integrity of hole "a" (*32*). Inspection of hole "a" itself showed that γ330Asp and γ364Asp are the two residues that contribute mostly to the local negative charge in the cavity so that in the γD364A structure the negative charge distribution inside the cavity is substantially decreased.

Plasmin Protection Assay. To assess binding of both calcium and GPRP in γ364Asp variants, we performed plasmin protection assays. In this assay, the addition of GPRP or calcium to the reaction limits the plasmin proteolysis of fibrinogen to fragment D_1. In the presence of EDTA or in the absence of GPRP, fragment D_1 is further cleaved to the smaller fragments D_2 and D_3. This assay also enabled us to determine whether GPRP binding observed in the crystal structure of γD364A also occurs in solution. As shown in Figure 6, essentially only fragment D_1 was seen in normal and variant fibrinogens at 5 mM $CaCl_2$ while in the presence of 5 mM EDTA, only the smaller fragments D_2 and D_3 were evident. Thus, with normal and variant fibrinogen, calcium protected fragment D_1 from further cleavage to D_2 and D_3, suggesting that calcium binding is not altered in these variants. In the presence of 4 or 20 mM GPRP, only the normal fibrinogen was completely protected from plasmin as evident from the presence of only fragment D_1. At both 4 and 20 mM GPRP, the γD364A digests contained fragments D_2 and D_3 and a significant level of D_1, demonstrating partial plasmin protection. Our crystal structure showed GPRP binding to hole "a" in γD364A; thus, we expected to see complete protection from plasmin digestion. Because γD364A showed partial degradation, we concluded that γD364A weakly binds GPRP. In contrast, fragments D_2 and D_3 were the only products of plasmin digestion with γD364H and γD364V fibrinogens, indicating complete loss of protection. We concluded that the extensive degradation of γD364H and γD364V indicates severely impaired holes "a" that are incapable of binding GPRP.

DISCUSSION

Although the interactions between γ364Asp and GPRP have been shown in several crystallographic studies of fibrinogen, the impact of substitution at this site was not fully appreciated at the molecular level. Our structural data showed GPRP bound to hole "a" in γD364A despite loss of the ionic interaction between the carboxylate side chain of γ364Asp and the free amino group of the peptide. Partial protection from plasmin proteolysis suggests GPRP binds to hole "a" in γD364A, although probably more weakly compared to normal. In contrast, the lack of protection and extensive degradation by plasmin imply GPRP does not bind either γD364H or γD364V. The protection conferred by calcium suggests the global structures of γD364H and γD364V are not markedly different from normal.

Previous studies with reptilase, an enzyme that cleaves only FpA, revealed normal FpA release in all γ364Asp variants, but polymerization was not evident by either turbidity or γ-γ dimer formation (*12*). These data indicate that these variants have incompetent holes "a" such that "A:a" interactions are not sufficient for polymerization. Moreover, laser tweezers experiments showed "A:a" interactions were undetectable in both γD364A and γD364H (*33*). On the basis of these functional data, we expected structural studies would show altered hole "a" in γD364A. Thus, we were surprised to find that γD364A can support GPRP binding to hole "a". The rfD-γD364A+GP structure showed that "A:a" interactions can occur. With both normal and γD364A fibrinogen capable of "A:a" interactions with only minor changes in hole "a", one would wonder why γD364A function is so impaired compared to normal. Questions then arise: how does γ364Asp impact "A:a" interactions and how does the loss of this acidic residue affect polymerization?

In the first three-dimensional structures showing the polymerization hole obtained from a 30 kDa C-terminal γ-chain fragment with and without GPRP peptide, Yee et al. and Pratt et al. (*24, 30*) showed large areas of negatively charged residues in and around hole "a" that result to a large molecular dipole moment. This cluster of negative charges interacts with the positively charged GPRP. The authors suggested that electrostatic steering may be a significant component of the fibrin polymerization mechanism that guides the alignment of fibrin monomers (*30*). Hole "a" is surrounded by negatively charged loops that bind calcium, and inside the cavity are two acidic residues γ330Asp and γ364Asp that give the hole a local negative charge. These residues may provide a local negative charge that attracts and facilitates binding of the positively charged knob "A" to hole "a". It is known from the amino acid sequence of fibrinogen that the molecule has considerably more negative than positive charges, with much of the excess negative charges located at the central domain (*34*). Removal of the two FpAs reduces the negative charge on the central domain from −8 to −1, but it is only after cleavage of FpB that the central domain actually assumes a net positive charge (*3*). It is reasonable to conclude then that the local negative charge on hole "a" reinforces electrostatic steering so that knobs "A" at the center of one molecule are able to find the holes at the terminal ends of another molecule during protofibril formation. The prolonged lag time during thrombin-catalyzed polymerization (*12*) suggests slow protofibril formation in

γD364A and may be a consequence of the diminished electrostatic steering due to the loss of the acidic γ364Asp.

The fact that GPRP confers only partial and not full protection to γD364A from plasmin degradation suggests a lower knob-hole affinity and less stable binding compared to normal. The salt bridge between the γ364Asp carboxylate and the free amino group of the knob strengthens "A:a" interactions and loss of such important ionic interaction may contribute to less stable "A:a" interactions in γD364A. Complete protection with calcium suggests that there are no global changes in the structures of γD364H and γD364V, but the complete loss of protection with GPRP suggests these altered holes "a" do not bind GPRP. Valine has a slightly bigger van der Waals volume (105 Å^3) than aspartic acid (91 Å^3) such that contact between the aliphatic side chain and the positively charged amino group of the knob is unfavorable. In the event of knob "A" binding, valine could undergo rotameric switch, but unlike aspartic acid or alanine, branching at the β-carbon limits its conformational freedom. These steric effects would highly destabilize "A:a" interactions in γD364V, in addition to the loss of the important electrostatic steering mediated by γ364Asp. For γD364H, the polar and bulky histidine (118 Å^3) may introduce rearrangements in neighboring residues. This may alter the shape and volume such that there is no reasonable polymerization hole present in γD364H. In addition to the loss of the electrostatic force that attracts and directs the knob to the hole, the substantial rearrangements in γD364H may preclude "A:a" interactions in this variant.

In summary, our biochemical and structural data showed that γ364Asp may not be required for knob "A" binding to hole "a" per se but substitution at this position alters electrostatic properties of hole "a" that are critical for normal fibrin polymerization.

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