Role of the Glycoprotein Ib-Binding A1 Repeat and the RGD Sequence in Platelet Adhesion to Human Recombinant von Willebrand Factor

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To assess the relative importance of the glycoprotein (GP) Ib binding domain and the RGDS binding site in platelet adhesion to isolated von Willebrand factor (vWF) and to collagen preincubated with vWF, we deleted the A1 domain yielding ΔA1-vWF and introduced an aspartate-to-glycine collagen preincubated with vWF, we deleted the A1 domain yielding ΔA1-vWF and introduced an aspartate-to-glycine substitution in the RGDS sequence by site-directed mutagenesis (RGGS-vWF). Recombinant ΔA1-vWF and RGGS-vWF, purified from transfected baby hamster kidney cells, were compared with recombinant wild-type vWF (WT-vWF) in platelet adhesion under static and flow conditions. Purified mutants were coated on glass or on a collagen type III surface and exposed to circulating blood in a perfusion system. Platelet adhesion under static condition, under flow conditions, and in vWF-dependent adhesion to collagen has an absolute requirement for GPIb-vWF interaction. The GPIb/IIa-vWF interaction is required for adhesion to coated vWF under flow conditions. Under static condition and vWF-dependent adhesion to collagen, platelet adhesion to RGGS-vWF is similar as to WT-vWF, but platelet spreading and aggregation are abolished.

The large, multimeric glycoprotein von Willebrand factor (vWF), is synthesized by endothelial cells and megakaryocytes and is released by endothelial cells into the plasma, as well as being deposited in the subendothelium. It is well established that vWF mediates the flow-dependent adhesion of platelets to vascular subendothelium after injury of the endothelial cell layer. In this process vWF acts as a bridge between substances in the exposed vessel wall and receptors on platelets. The subendothelium contains a variety of extracellular matrix components, such as fibrillar/nonfibrillar collagens, microfibrils, and proteoglycans; all may provide binding sites for vWF. Although collagen may not be the first recognition site of vWF in subendothelium, it certainly does play an important role in platelet adhesion to deeper layers of the vessel wall. The molecular mechanisms responsible for these interactions are complex and not completely understood.

Also, vWF binds to two distinct platelet receptors: the glycoprotein (GP) Ib/IX receptor and the GPIb/IIa receptor. In vitro, the GPIb-vWF interaction can only be studied in the presence of modulators such as ristocetin or botrocetin, because soluble vWF has no affinity for GPIb. Although collagen may not be the first recognition site of vWF in subendothelium, it certainly does play an important role in platelet adhesion to deeper layers of the vessel wall. The molecular mechanisms responsible for these interactions are complex and not completely understood.

The vWF cDNA has been cloned and the protein has been sequenced showing a characteristic composition of repeating homologous domains: the A, B, C, and D domains. To date, several binding sites have been localized to discrete domains on the mature subunit of vWF, eg, binding sites for GPIb, GPIb/IIa, collagen, and perhaps other subendothelial substances. Studies with proteolytic fragments of vWF, recombinant A1 domains, or monoclonal antibodies (MoAbs) against vWF showed that the GPIb binding site is located on the A1 domain. We have confirmed and extended these data by showing that a deletion mutant that lacks the A1 domain (residues 478 to 716) did not bind to ristocetin-treated platelets. By using MoAbs specific for GPIb or GPIb/IIa, Chow et al showed that shear stress-induced aggregation was completely or at least partially inhibited. Recently, Danton et al showed by using the same MoAbs that platelet adhesion to purified vWF under flow was absent using either of the two MoAbs. Using MoAbs directed against the A1 domain and the RGD sequence, Pessinaud et al showed that interactions with both platelet receptors were involved when collagen was used as the adhesive surface. Recombinant (r) vWFs that had a point mutation in the RGD sequence could not compete with normal vWF for binding to adenosine diphosphate (ADP)- or thrombin-stimulated platelets.

In this study, we examined the vWF interactions with platelet receptors under static and flow conditions using mutants of vWF. We used the ΔA1-vWF as described previously and made a point mutation in the RGDS sequence: Asp-1746 was replaced by Gly, yielding RGGS-vWF. The r-vWFs were transfected in baby hamster kidney (BHK) cells, and stable cell lines were established. The mutants were coated on glass or on a collagen type III surface and exposed to circulating blood in a perfusion system. Our results indicate that in a static system, GPIb-vWF interaction is necessary and sufficient for platelet adhesion. Under flow conditions, two platelet receptors, GPIb and GPIb/IIa, are both necessary. For vWF-mediated adhesion to collagen type III, GPIb is the only receptor required for adhesion. Absence of interaction between vWF and GPIb/IIa leads to absence of platelet aggregate formation.

MATERIALS AND METHODS

Materials. Restriction enzymes and DNA modifying enzymes were from New England Biolabs (Beverly, MA) or Pharmacia (Uppsala, Sweden). Synthetic oligonucleotides were prepared on an Applied Biosystems model 380B DNA synthesizer (Foster City, CA) at the Protein Chemistry Laboratory of the University Medical Center Utrecht, Utrecht, The Netherlands.

METHODS

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Construction of the expression vector. To obtain stable expression of rVWF in mammalian cells, an expression vector was constructed in the following way. An EcoRI fragment containing the full-length cDNA of VWF was excised from pSP8000-VWF. The fragment was isolated, and the cohesive ends were filled with the Klenow fragment of Escherichia coli DNA polymerase I. The fragment was ligated according to the method described by Pecenka et al.15 into the Smal I site of the mammalian expression vector pNUT,16 and the correct orientation was determined using restriction enzyme digests. The construct was named pNUT-VWF.

Site-directed mutagenesis. The preparation of the ΔA1-VWF plasmid has been described.29 The expression vector pSV2-ΔA1-VWF was digested with the restriction enzymes Eag I and EcoRV. The Eag I-EcoRV fragment containing the deletion of the coding sequence for amino acids 478 to 716, was cloned into pNUT-VWF, yielding pNUT-ΔA1-VWF. RGGS-VWF was made by changing the nucleotide positions 7527 and 7528 from AC to GA, resulting in the substitution of amino acid Asp(D)-1746 in Gly(G)-1746. Site-directed mutagenesis was performed with the oligonucleotide 5'-CGG GGG GG*A*TCC CAG TCT-3'. The mutant VWF cDNA insert was sequenced according to the dideoxy chain-termination method of Sanger et al.27 The desired mutation in the RGD sequence (1744 to 1746) of VWF was present. One G to T transition had occurred at base 4465, which did not affect the amino acid sequence. An Eag I-EcoRV fragment containing the cDNA mutations was subcloned into the pNUT-VWF expression vector as described above for ΔA1-VWF, yielding pNUT-RGGS-VWF for expression in BHK cells.

Transfection of BHK cells and selection of stable transformants. BHK-21 cells were transfected using the calcium phosphate method as described by Graham and Van der Eb.32 Briefly, cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1; Gibco, Paisley, UK) supplemented with penicillin G (100 U/mL; Brocades Pharma, Leiderdorp, The Netherlands), streptomycin sulfate (100 μg/mL; Biochemie, Vienna, Austria), 2 mmol/L L-glutamine (GIBCO, Paisley, UK) and 5% heat-inactivated fetal calf serum. After maintaining the cells in complete medium for 2 days, selection was initiated by adding 100 μmol/L methotrexate (MTX; Sigma, St. Louis, MO) before the gel filtration run. Before concentration, the culture was kept in PBS until perfusion. From studies with 125I-vWF, we estimated the highest ratio of ristocetin cofactor activity versus vWF antigen concentration, were used in adhesion studies. In the case of ΔA1-VWF, which has no ristocetin cofactor activity, fractions 2 and 3 with the highest VWF antigen concentration were used.

Determination of the multimeric structure. The multimeric structure of VWF was assayed using the Pharmacia Phast Gel System (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described by Lawrie et al.16 Briefly, samples diluted in 10 mmol/L TRIS/HCl, 1 mmol/L EDTA, 2% sodium dodecyl sulfate (SDS), 8 μmol/L urea, and 0.05% bromophenol blue, pH 8.0, were applied to a 1.7% agarose gel (LE; Seakem, FMC Bioproducts, Rockland, ME) in 0.5 mol/L TRIS/HCl pH 8.8, 0.1% SDS, with a stacking gel consisting of 0.8% agarose (HGT; Seakem) in 0.125 mol/L TRIS/HCl pH 6.8 and 0.1% SDS. After electrophoresis, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane (ImmunoP; Millipore) by diffusion blotting for 1 hour at 60°C. The membrane was then blocked with 5% non-fat dry milk protein solution (Protifar; Nutricia, Zoetermeer, The Netherlands) for 1 hour at room temperature.

Coating and spraying of the cover slips. Glass coverslips (18 x 18 mm; Menzel Gisler, Braunschweig, Germany) were cleaned overnight by a chromium trioxide solution and rinsed with distilled water before coating. Coating was performed by incubating the cover slips with purified VWF (5 μg/mL) for 1 hour at room temperature, followed by a 1-hour incubation with 4% human albumin (Behringwerke AG, Marburg, Germany) solution in PBS to block further protein binding to the glass surface. After coating, the cover slips were kept in PBS until perfusion. From studies with 125I-VWF, we estimated the amount of VWF present on the cover slip after incubation with a solution of 5 μg/mL as 30 ng/cm² (data not shown).

Static adhesion assay. Static adhesion experiments were performed in a 24-well microtitre plate (Costar, Cambridge, MA) using circular coverslips (Knittel Gisler, Braunschweig, Germany) with a surface area of 1.1 cm².
Platelet-rich plasma (PRP) was prepared from low-molecular-weight heparin (LMWH: Fragmin; Kabi Pharmacia, Stockholm, Sweden) blood by centrifugation (200g, 10 minutes, 20°C). The platelet count was adjusted with platelet-poor plasma (PPP) to 200,000/μL, and 400 μL was added to a well. Platelets were allowed to adhere for 45 minutes at 37°C. Subsequently, the coverslips were rinsed with PBS, fixed with 0.5% glutaraldehyde, dehydrated in methanol, and stained with May-Grünwald-Giemsa. This experimental approach allowed us to evaluate the interaction of platelets with immobilized vWF in a plasma environment with normal concentrations of divergent cations, essential for ligand-binding function of GPIIb/IIIa, and with minimal manipulation of the platelets before use.

Perfusate. Whole blood was obtained from healthy volunteer donors who had taken no aspirin or other platelet function inhibitors in the preceding week and was anticoagulated with 1/10 vol 200 U/mL LMWH (Fragmin) in 0.15 mol/L NaCl (LMWH-blood). Recently, Baruch et al. reported that LMWH does not interact with vWF.

Reconstituted perfusates were prepared as follows. PRP was obtained from whole blood by centrifugation (10 minutes at 200g, 20°C). One volume of Krebs-Ringer buffer (4 mmol/L KCl, 107 mmol/L NaCl, 20 mmol/L NaHCO₃, and 2 mmol/L Na₂SO₃, containing 19 mmol/L sodium citrate, pH 5.0) was added to 1 vol of PRP. The final pH was about 7. The platelet pellet was obtained by centrifugation (10 minutes at 500g, 20°C). The platelet pellet was resuspended in Krebs-Ringer buffer, pH 6.0, and washed twice by centrifugation (10 minutes at 500g). After the second wash, platelets were resuspended to a platelet count of 190,000/μL in a human albumin solution (HAS; 4% human albumin in Krebs-Ringer buffer without citrate, pH 7.35, containing 20 U/mL LMWH, 5 mmol/L α-D-glucose, and 2.5 mmol/L CaCl₂). Red blood cells were washed three times with PBS containing 5 mmol/L α-D-glucose (2000g at 20°C, twice for 5 minutes and the last time for 15 minutes). Washed red blood cells were added in a volume fraction of 40% of total volume at 5 minutes before perfusion.

Perfusion procedure. The flat chamber perfusion system as developed by Sakariassen et al. was used for all perfusion experiments. The perfusate was prewarmed for 5 minutes at 37°C and was subsequently recirculated at different wall shear rates through the chamber for 5 minutes at the same temperature. After perfusion, the coverslip was washed with HEPES buffer (150 mmol/L NaCl, 10 mmol/L HEPES, pH 7.35), fixed by 0.5% glutaraldehyde in PBS, dehydrated in methanol, and stained with May-Grünwald-Giemsa. Platelet adhesion was evaluated with a light microscope at 1,000× magnification, connected to an Image Analyzer (AMS 40-10, Saffron Walden, UK). Evaluation was performed of 30 fields, perpendicular to the flow axis, in the center of each glass coverslip. Platelet adhesion was expressed as percent surface covered with platelets.

Statistical analysis. The mean ± SD was calculated for experiments based on three or more determinations. The significance of differences in each group was evaluated with Student’s t-test. Probability values less than .05 were considered significant.

RESULTS

Expression of rvWF. In our previous studies, WT-vWF, ΔA1-vWF, and RGG5-vWF were transfected into COS-l cells to study binding to heparin, collagen, and platelets. For the perfusion experiments described in the current report, much more rvWF is needed than COS cells can produce. For this reason, the rvWF constructs were transfected in BHK cells, and stable cell lines were established after selection with 100 μmol/L MTX. The levels of expression in the cell culture supernatant varied from 1.0 to 1.5 μg/mL for WT-vWF to 2.0 to 4.0 μg/mL for ΔA1-vWF and RGG5-vWF, comparable with those reported for other rvWF-producing cell lines. These levels indicated that the mutations did not influence the synthesis or secretion of the rvWFs. For analysis of subunit composition of the rvWFs, the cell culture supernatants were assayed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions followed by transfer to a PVDF membrane (Fig 1A). All cell lines had a large amount of pro-vWF compared with the mature subunit, an observation also made by other investigators who have established stable cell lines of vWF. The band shifts to lower molecular weight in the case of the
ΔA1-vWF (Fig 1A) clearly indicated the absence of the A1 domain. Analysis of the multimeric structure of the rvWF mutants (Fig 1B) showed no difference between WT-vWF and RGGS-vWF, suggesting that the point mutation Asp1746Gly did not influence the multimerization of vWF. The ΔA1-vWF mutant lacked the very high-molecular-weight multimers. Deleting the A1 domain evidently resulted in an rvWF molecule unable to form the higher-molecular-weight multimers, similar to our previous findings in COS-1 cells.*

Characterization of rvWF. The rvWF mutants were purified from culture medium using gel filtration. The concentration of rvWF present in the fractions varied from 5 to 10 μg/ml. As we described previously, the ΔA1-vWF does not bind to heparin or to GPIb. It has normal binding to factor VIII (FVIII) and binds to collagen type III and thrombin-activated platelets. We also found that the point mutation Asp1746Gly did not influence binding to heparin, collagen type III, and FVIII. In the current study, we examined the effect of the mutation on binding to platelet GPIIb/IIIa using an ELISA procedure in which we coated purified GPIIb/IIIa to an ELISA well. As shown in Fig 2, the binding of WT-vWF and ΔA1-vWF to GPIIb/IIIa was saturable, whereas RGGS-vWF showed no binding at all. The same results were obtained when the vWF mutants were immobilized on the plates and GPIIb/IIIa was in solution (data not shown). The absence of RGGS-vWF binding also indicates that vWF binding to GPIIb/IIIa is not mediated by interaction with the RGD sequence present in the propeptide. We cannot exclude that it may play a role as a secondary binding site, but this seems unlikely in view of the similarity of binding of plasma vWF, which lacks the propeptide, and WT-vWF, of which part of the molecules still possess the propeptide (results not shown).

Platelet adhesion to plasma and rvWFs under static condition. Unstimulated platelets in PRP adhered selectively to surfaces coated with 5 μg/mL purified plasma-vWF (PL-vWF), WT-vWF, and RGGS-vWF, but not to surfaces coated with ΔA1-vWF and human albumin (Table 1). These results indicated that the loss of the A1 domain resulted in an rvWF unable to support platelet adhesion under static condition. The control surfaces, plasma-vWF and WT-vWF, induced platelet attachment and spreading (Fig 3A). Contact and dendritic platelets but no spread platelets were observed when the surface was incubated with RGGS-vWF (Fig 3B). This result indicates that under static condition, the interaction of RGGS-vWF with one receptor, GPIb, is sufficient for platelet adhesion.

Platelet adhesion to PL-vWF and rvWFs under flow conditions. PL-vWF, WT-vWF, ΔA1-vWF, and RGGS-vWF were adsorbed to glass coverslips at 5 μg/mL. These coverslips were then exposed to whole blood anticoagulated with LMWH using two wall shear rates: 300 s\(^{-1}\) and 1,600 s\(^{-1}\). By blocking of the uncoated glass with human albumin, vWF from the plasma cannot coat to this surface anymore; thus, platelet adhesion to coated vWF can be performed

Table 1. Platelet Adhesion to vWF Under Static Conditions

<table>
<thead>
<tr>
<th>Surface</th>
<th>% Coverage</th>
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<tbody>
<tr>
<td>PL-vWF</td>
<td>3.7 ± 0.5</td>
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<tr>
<td>WT-vWF</td>
<td>4.6 ± 0.5  (NS)</td>
</tr>
<tr>
<td>ΔA1-vWF</td>
<td>0.1 ± 0.1t</td>
</tr>
<tr>
<td>RGGS-vWF</td>
<td>2.0 ± 0.7*</td>
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<tr>
<td>HAS</td>
<td>0.1 ± 0.1t</td>
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</table>

PL-vWF, rvWFs (WT, ΔA1, and RGGS), and human albumin (HAS) were coated to glass coverslips after incubation with PRP for 45 minutes at 37°C. Nonadherent platelets were then removed by aspiration. After washing with PBS, the coverslips were fixed and stained. Adhesion was evaluated as the percentage surface covered with platelets and measured by automatized image analysis. Results are from one experiment of three similar experiments with blood of different donors and are expressed as mean ± SD of three coverslips per experiment.

Abbreviation: NS, not significant.
* P < .01 compared with WT-vWF; NS compared with PL-vWF.
† P < .001 compared with PL-vWF or WT-vWF.
using whole blood. No changes in the amount of adsorbed protein were found after perfusion for 5 minutes at shear rates between 300 and 1,600 sec⁻¹ (data not shown). The data (Table 2) showed an increased platelet adhesion to immobilized PL-vWF and WT-vWF with increasing shear rate, confirming previous results. The adhesion to WT-vWF was greater than to PL-vWF for unexplained reasons. The ΔA1-vWF mutant, which lacks the GPIb binding domain, gave no adhesion at both shear rates. There was also no platelet adhesion to RGGS-vWF or to human albumin at both shear rates.

From previous studies in our laboratory, we estimated that the amount of vWF present on the coverslip after incubation with vWF at 5 μg/mL was 30 ng/cm². The results from Table 2 provide strong evidence that both the A1 domain and the RGD sequence are necessary for platelet adhesion under flow conditions. This means that, under flow, there are two receptors, GPIb and GPIIb/IIIa, involved in platelet adhesion to purified immobilized vWF.

Table 3. Platelet Adhesion to Collagen Type III Using vWD Blood

<table>
<thead>
<tr>
<th>Surface</th>
<th>% Coverage</th>
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<tr>
<td>WT-vWF</td>
<td>14.2 ± 2.4</td>
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<tr>
<td>ΔA1-vWF</td>
<td>1.4 ± 0.8*</td>
</tr>
<tr>
<td>RGGS-vWF</td>
<td>13.5 ± 1.9 (NS)</td>
</tr>
<tr>
<td>HAS</td>
<td>1.6 ± 0.6*</td>
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Recombinant vWFs (WT, ΔA1, RGGS) were coated to glass coverslips that were preincubated with collagen type III. As a control, HAS was coated to collagen-treated coverslips. The coverslips were exposed to shear rate of 1,600 sec⁻¹ using LMWH whole blood from a patient with severe von Willebrand disease as the perfusate. Results are from one of two similar experiments with blood of two different patients, and are expressed as mean ± SD of three coverslips.

* P < .001 compared with WT-vWF.
function in the adhesion process by binding to vWF.

Especially under conditions of relatively high shear rates, vWF is an adhesive protein. An approximation of the in vivo vWF interactions can be obtained using the flat-chamber perfusion system as developed by Sakariassen et al. Numerous studies have shown that, in this system, conditions are provided that simulate those that may act in the circulation. In the current study, we analyzed the attachment and spreading of platelets under static and flow conditions using vWF on which the GPIb and GPIIb/IIIa receptor-binding sites had been deleted (ie, mutated). Adhesion studies using surfaces that were incubated with vWF lead to the following conclusions: (1) platelet adhesion under static condition, flow conditions, and vWF-dependent adhesion to collagen has an absolute requirement for GPIb-vWF interaction; and (2) GPIIb/IIIa-RGD interaction is absolutely required for adhesion under flow conditions. Under static condition, adhesion to vWF was not affected, but platelets did not spread. The vWF-dependent adhesion to collagen was somewhat decreased when the RGD site was mutated, and platelet spreading and aggregation was abolished.

These studies confirm and extend the data of Savage et al who showed that platelet interaction with surface-coated vWF was primarily mediated by the GPIb-vWF interaction, which was then followed by the GPIIb/IIIa-RGD interaction required for platelet spreading and firm attachment. Their studies performed under static condition and studying platelet adhesion over a period of 45 minutes suggested that GPIIb/IIIa-RGD interaction would be necessary to obtain adhesion that would be resistant to shear. This is, indeed, what we found in our more physiologic perfusion studies. The observation that the GPIb-vWF interaction is critical under static condition, which we confirm here, reemphasizes that it is a conformational change in vWF induced by adsorption to a surface that allows this interaction to occur and not, as also has been suggested, the effect of shear. The results obtained in adhesion to collagen deserve special attention. Recent studies from our laboratory confirming earlier studies show that GPIa/IIa (VLA2) is an important receptor for adhesion to collagen under flow conditions. Previous studies have also shown that this adhesion is vWF-dependent.

**DISCUSSION**

Adhesion receptors on platelets play an important role in platelet adhesion at sites of vascular injury. Among these, GPIb and GPIIb/IIIa have been shown to fulfill a pivotal role in platelet adhesion to collagen type III preincubated with (A) WT-vWF and (B) RGGS-vWF. Original magnification × 250.

**Fig 4. Morphology of platelet adhesion to collagen type III using severe von Willebrand disease blood as the perfusate: adhesion to collagen type III preincubated with (A) WT-vWF and (B) RGGS-vWF.**

Adhesions using these normal platelets are shown in Table 4. PL-vWF and WT-vWF resulted in about the same percentage surface coverage. Platelet adhesion to RGGS-vWF was somewhat lower in contrast with the results with the von Willebrand disease type 3 blood in Table 3; and ΔA1-vWF and human albumin gave no adhesion at all. The difference may be caused by the slight activation of platelets during the preparation of reconstituted blood. Activation of platelets may lead to downregulation of surface GPIb, and this may cause a decreased vWF-mediated adhesion to collagen type III, which becomes visible for RGGS-vWF because the GPIb-vWF is the single interaction occurring between platelets and vWF. The morphologies of platelet adhesion to PL-, WT-, and RGGS-vWF were the same as those observed in the previous experiment using von Willebrand disease type 3 blood in Table 3. These results indicate that, under the experimental conditions, PL-vWF does not play a role in platelet adhesion to collagen surfaces preincubated with different vWFs.

**Table 4. Platelet Adhesion to Collagen Type III Using Normal Platelets**

<table>
<thead>
<tr>
<th>Surface</th>
<th>% Coverage</th>
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<tr>
<td>PL-vWF</td>
<td>6.3 ± 1.1</td>
</tr>
<tr>
<td>WT-vWF</td>
<td>9.6 ± 1.2 (NS)</td>
</tr>
<tr>
<td>ΔA1-vWF</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>RGGS-vWF</td>
<td>2.9 ± 0.31</td>
</tr>
<tr>
<td>HAS</td>
<td>0.1 ± 0.1*</td>
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</table>

PL-vWF and vWFs (WT, ΔA1, RGGS) were coated to glass coverslips that were preincubated with collagen type III. As a control, a 4% HAS was coated to collagen-treated coverslips. The coverslips were exposed to normal platelets reconstituted in 4% human albumin in Krebs-Ringer buffer at a shear rate of 1,600 s⁻¹. Results are from one of three similar experiments with blood of different donors, and are expressed as mean ± SD of four coverslips obtained in two runs, with two coverslips per run.

* P < .01 compared with PL-vWF.

† P < .001 compared with WT-vWF; NS compared with PL-vWF.
PLATELET ADHESION TO VON WILLEBRAND FACTOR

Our current studies using ΔA1-vWF indirectly show that the GPIb-vWF interaction is critical for platelet adhesion to collagen. We also show that the GPIb-IIIa-vWF interaction plays an important role in subsequent platelet aggregation. This observation is an extension of the concepts worked out by Savage et al. who showed that interaction of platelets with vWF via GPIb caused activation of GPIb/IIIa, leading to platelet spreading and firm attachment. Our studies show that interaction of platelets with vWF on collagen via GPIb/IIIa is required for platelet aggregation. The multiple interactions involved in adhesion to collagen are GPIa/IIa-collagen, GPIb-vWF, and GPIIb/IIIa-vWF. Evidently, all are required for platelet aggregate formation on collagen under flow.

The rvWF produced by stable BHK cell lines contains a large proportion of the propolypeptide form, an observation also made by others. The reason for the incomplete processing remains obscure and is probably caused by the high amount of rvWF produced, saturating the processing enzyme furin. The multimeric structure of WT-vWF and RGGS-vWF is comparable with that of PL-vWF, with a high amount of high-molecular-weight multimers. Deletion of the A1 domain resulted in an rvWF molecule unable to form the higher-molecular-weight multimers in BHK cells, an observation also made in our previous studies using COS cells.

Although the propolypeptide was present in our rvWF molecules, we found no evidence of any contribution of the propolypeptide RGD sequence in binding to platelet GPIb/IIIa. The RGGS-vWF mutant did not bind to isolated GPIb/IIIa in an ELISA system. Platelets adhering to immobilized RGGS-vWF did not spread, indicating that there was no interaction with the GPIb/IIIa receptor on platelets. Beacham et al. also did not detect any interference of the RGD sequence of the propolypeptide in inhibition studies using ADP- or thrombin-stimulated platelets and rvWF mutants.

Perfusion studies using collagen type III as the adhesive surface show that, by preincubation with ΔA1-vWF, the primary adhesion is mediated via vWF already present on the collagen surface. The presence of PL-vWF does not interfere in this adhesion process.

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Role of the glycoprotein Ib-binding A1 repeat and the RGD sequence in platelet adhesion to human recombinant von Willebrand factor

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