Comparative Medicine
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Vol 62, No 4
August 2012
Pages 291–297

Original Research

Cloning of Porcine Platelet Glycoprotein Ibα and Comparison with the Human Homolog

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Glycoprotein Ib–IX–V (GPIb–IX–V) is a platelet adhesion receptor complex that initiates platelet aggregation. Glycoprotein Ibα (GPIbα) is the central component of the GPIb–IX–V complex, anchoring the complex to the cytoskeleton and harboring the binding site for von Willebrand factor (vWF). Previous studies suggest that the coagulation function in pigs differs from that in humans, especially with respect to the interaction between vWF and platelets. However, we have little knowledge about the function of porcine platelets, which is important with regard to studies of cardiovascular disease, clotting, and surgery that use pigs as animal models. To extend this information, we cloned and analyzed the porcine GPIbα sequence. Porcine GPIbα contains 1891 nucleotides and includes an open reading frame that encodes 627 amino acids. The nucleotide sequence showed 67% identity with human GPIbα, whereas the deduced amino acid sequences were 59% identical. The vWF binding domain shares the highest identity among different species, whereas the PEST domain shows variations. Evaluation of platelet function by using ristocetin-induced platelet aggregation revealed remarkably lower levels of aggregation in porcine than human platelets. According to the sequence analysis and platelet aggregation tests, we propose that the function of GPIbα, especially regarding the ristocetin–vWF–GPIbα interaction, differs between pigs and humans. This characterization of porcine GPIbα will enhance our knowledge of the porcine coagulation system.

Abbreviations: GPIbα, glycoprotein Ibα; vWF, von Willebrand factor.

Glycoprotein (GP) Ib–IX–V is one of the major adhesive receptors expressed on the surface of circulating platelets and is essential for platelet adhesion and clot formation at sites of vascular injury.[2] Platelet adhesion in high-shear areas is initiated by GPIbα, a subunit of the GPIb–IX–V complex, via binding to von Willebrand factor (vWF), a multimeric adhesive protein associated with collagen in the vessel wall.[3,13,27] After GPIbα-dependent adhesion to vWF, platelets become activated and undergo cytoskeletal rearrangements associated with shape changes, spreading, and the secretion of platelet agonists that amplify the platelet aggregation and activation mediated by platelet integrin $\alpha_{IIb}\beta_3$.[1]

The GPIb–IX–V complex consists of 4 transmembrane subunits—GPIbα, GPIbβ, GP IX, and GP V—which are present at a ratio of 2:2:2:1.[26] The entire ligand-binding capacity of the GPIb–IX–V complex is situated in the N-terminal globular region (amino acids 1 through 282) of GPIbα.[28] Mutations in GPIbα lead to Bernard–Soulier syndrome and pseudo-von Willebrand disease.[15,24] Thrombi that cause complications in arterial thrombosis are associated with GPIb–IX–V, especially GPIbα.[21] Because the interactions between GPIbα and its ligand are critical to the vascular processes of thrombosis and inflammation, the complex is under intense scrutiny as a potential therapeutic target.[29]

Pigs share many physiologic and anatomic similarities with humans and offer several breeding and handling advantages relative to nonhuman primates, making the pig an optimal species for preclinical experimentation. During the last several years, porcine animal models have gained a great deal of importance[23,30] in cardiovascular diseases,[6,33] ischemia–reperfusion injury,[10] transplant surgery, and many other areas of biomedical research.[17] In particular, the pig has been identified as an ideal cell, tissue, and organ donor for xenotransplantation. Because differences exist between species, it is necessary to take the physiologic differences between pigs and humans into account when developing animal models and when analyzing the results obtained by using these models.

Our early studies revealed differences in the process of coagulation between pigs and humans.[5] Currently we know little about which functions of platelets are conserved between species or about porcine GPIb–IX–V and its differences from the human complex. In the current study, we cloned the coding sequences of porcine GPIbα and compared its nucleotide sequence, deduced protein sequence, and 3D structure model with those of human GPIbα, focusing on important functional domains and vWF interaction sites. We also investigated the ability of porcine platelets to be agglutinated or activated when treated with ristocetin. This work represents a step toward understanding the value and limitations of the pig as a preclinical model for coagulation-related studies.

Received: 30 Jan 2012. Revision requested: 26 Feb 2012. Accepted: 31 Mar 2012.
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Figure 1. Nucleotide and deduced amino acid sequences of porcine GPIbα (GenBank accession no., FJ228700) are shown. Porcine GPIbα contains 1891 nucleotides and includes an open reading frame that encodes 627 amino acids. The initiation codon (ATG) and end codon (TAG) are underlined. The starting and ending points of each domain are indicated by double lines. The signal peptide comprises amino acid residues –16 through 0, the vWF-A1 binding domain is amino acids 1 through 280, the PEST domain comprises amino acids 281 through 477, and the transmembrane domain includes amino acids 496 through 523. Two potential N-glycosylation sites (N) predicted by the NetNGlyc 1.0 program (N19 and N468) are boxed.

Materials and Methods

Reagents. The following materials were used: the PureGen DNA Isolation Kit (Gentra Systems, Minneapolis, MN); TaKaRa Ex *Taq* and TaKaRa LA *Taq* (TaKaRa Bio, Tokyo, Japan); the EZNA Gel Extraction and EZNA Plasmid Extract kits (Omega Bio-Tek, Norcross, GA); the pMD18-T vector kit (TaKaRa Bio); ristocetin (Helena, Beaumont, TX); and SYBR Gold (Molecular Probes, Invitrogen, Eugene, OR).

Animals. Chinese Guizhou miniature pigs (*Sus scrofa*) were obtained from the Agriculture Institute of Guizhou University (Guiyang City, Guizhou, China), housed individually at the Animal Center of West China Hospital (Chengdu, China), and fed and watered ad libitum. All of the pigs were free of specific pathogenic microorganisms including hog cholera virus, pseudorabies virus, pathogenic dermal fungi, *Mycobacterium tuberculosis*, *Brucella* spp., *Leptospira* spp., *Salmonella* spp., and *Shigella* spp. All procedures in this study were in accordance with the *Guide for the Care and Use of Laboratory Animals*.[14] The experimental treatment of the animals was reviewed and approved by the Animal Care and Welfare Committee of West China Hospital, Sichuan University.

DNA isolation from porcine peripheral blood. Pigs were anesthetized (ketamine, 15 mg/kg IM), and whole blood samples were collected from the precaval vein by using heparin as an anticoagulant. DNA was extracted from whole blood by using the PureGen DNA Isolation Kit (Gentra Systems) according to the manufacturer's instructions. The quantity and integrity of the DNA were determined by spectrophotometry (model DU800, Beckman Coulter, Brea, CA) and gel electrophoresis.

Cloning of GPIbα. Because of the paucity of introns within GPIbα and because this gene contains virtually the entire coding

Table 1. DNA sequence and amino acid identity (%) of the domains of porcine GPIbα compared with human, monkey, bovine, and mouse homologs

	Human (%)	Monkey (%)	Bovine (%)	Mouse (%)
DNA sequence	67.00	66.70	70.78	65.83
Deduced AA sequence	58.84	56.55	60.26	59.41
VWF binding domain	68.18	69.70	71.02	72.00
PEST domain	34.86	33.51	48.63	29.19

Figure 2. Comparison of vWF binding sites in pigs and humans. The amino acid residues that comprise the specific vWF binding site of GPIbα[13] (black dots) include Ser11, His12, Glu14, Asn16, His37,Glu128, Lys152, Asp175, Thr176, Phe199, Glu225, Asn226, Tyr228, and Ser241. Among the amino acids involved in this site, 3 residues (blue boxes) differ between pigs and humans.

region within a single exon,[31] we designed the primers based on the genomic DNA sequences of GPIbα that are conserved among dogs, cattle, and humans as obtained from GenBank. The primers were designed by using Primer Premier 5 software (Primer Biosoft International, Palo Alto, CA). The primer sequences were 5′ GGT CAC CAT GCC CCT CCT CCT 3′ and 5′ TCA GAG GCT GTG GCC AGA GTA CC 3′. PCR amplification was performed by using Taq polymerase (Takara Bio) as follows: 94 °C for 20 min; 30 cycles of 94 °C for 1 min, annealing at 60 °C for 1 min, and 72 °C for 3 min; and a final extension step at 72 °C for 10 min. The amplified fragments were separated on a 1.5% agarose gel and visualized using SYBR Gold (Molecular Probes–Invitrogen) staining. After excision from the gel, the PCR products were cloned into the pMD18-T vector by using the TA-cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. The cloned cDNAs were verified by sequencing (Invitrogen, Eugene, OR).

Sequence analysis and alignments. Multiple-sequence alignments and further analysis of the DNA sequences were performed by using DNAMAN version 6 (DNAStar, Madison, WI). The BLASTN and BLASTX programs from NCBI (http://www.ncbi.nlm.nih.gov) were used to align the porcine sequence we obtained against the sequences in various databases.

For further analysis of the porcine GPIbα protein sequence, several online ExPASy Proteomics tools (http://au.expasy.org/tools/) were used, including the Simple Modular Architecture Research Tool (SMART), SignalP, OGPET, and NetNGlyc, to predict functional domains, signal peptide cleavage sites, and glycosylation sites. Homology modeling of the 3D protein structure of porcine GPIbα was performed by using the Swiss Institute of Bioinformatics program SWISS–MODEL (http://swissmodel.expasy.org/). The figures were drawn by using DeepView software (Swiss PDP Viewer, www.expasy.org/spdbv).

Platelet aggregation studies. Whole blood was collected from healthy human volunteers (n = 6) and minipigs (n = 6) by using 3.2% (w/v) trisodium citrate as an anticoagulant. The use of human subjects was approved by the ethics committee of West China Hospital, Sichuan University, and each subject gave their written informed consent. Platelet-rich plasma was prepared by centrifuging the anticoagulated samples at 160 × g for 20 min at room temperature, and the supernatant was collected and used as platelet-rich plasma. The material remaining after removal of the supernatant was centrifuged at 1000 × g for 10 min at room temperature for its use as platelet-poor plasma and a control. Platelet aggregation after the addition of ristocetin was recorded by using an aggregometer (model 700, Whole Blood Optical Lumi Aggregometer, Chrono-Log, Havertown, PA).

Results

Cloning the cDNA of porcine GPIbα. Positive clones were enriched and sequenced. The GPIbα sequence contained 1891 bp, with a complete open reading frame encoding 627 amino acids. The sequence of the full-length porcine GPIbα gene (Figure 1) was submitted to GenBank (GenBank accession no., FJ228700).

Sequence analysis and comparison with human GPIbα. Comparing the nucleotide sequence of porcine GPIbα with that of human GPIbα revealed that that the homology of the DNA sequences was 67%. The identity of predicted amino acid sequences was only 59%. The similarity of individual domains of GPIbα at the amino acid level differed among the 5 species evaluated (porcine, bovine, human, monkey, and mouse ; Table 1), with the vWF binding domain showing the highest similarity among the 5 species.

We used the SMART program to deduce the functional domains of porcine GPIbα as follows: signal peptide, amino acids –16 to 0; a leucine-rich repeat N-terminal domain, amino acids 3 through 35; 4 leucine-rich repeat outlier domains (amino acids, 54 through 75, 76 through 98, 147 through 170, and 171 through 194); 2 leucine-rich repeat domains belonging to the TYP subfamily, amino acids 99 through 121 and 123 through 146; a leucine-rich repeat C-terminal domain, amino acids 205 through 264; and a transmembrane domain, amino acids 498 through 520. The functional domains of porcine GPIbα predicted by the SMART program are similar to the human GPIbα domains. Compared with human GPIbα, the porcine protein has an extra leucine-rich repeat domain, located at amino acid residues 54 through 75.

Sequence analysis of the glycosylation sites by using NetNGlyc 1.0 software revealed 2 potential N-glycosylation sites (N19 and N468) in the porcine GPIbα protein (Figure 1). The predicted glycosylation sites in human GPIbα were N21 and N159. The online program OGPET version 1.0 revealed that porcine GPIbα had no

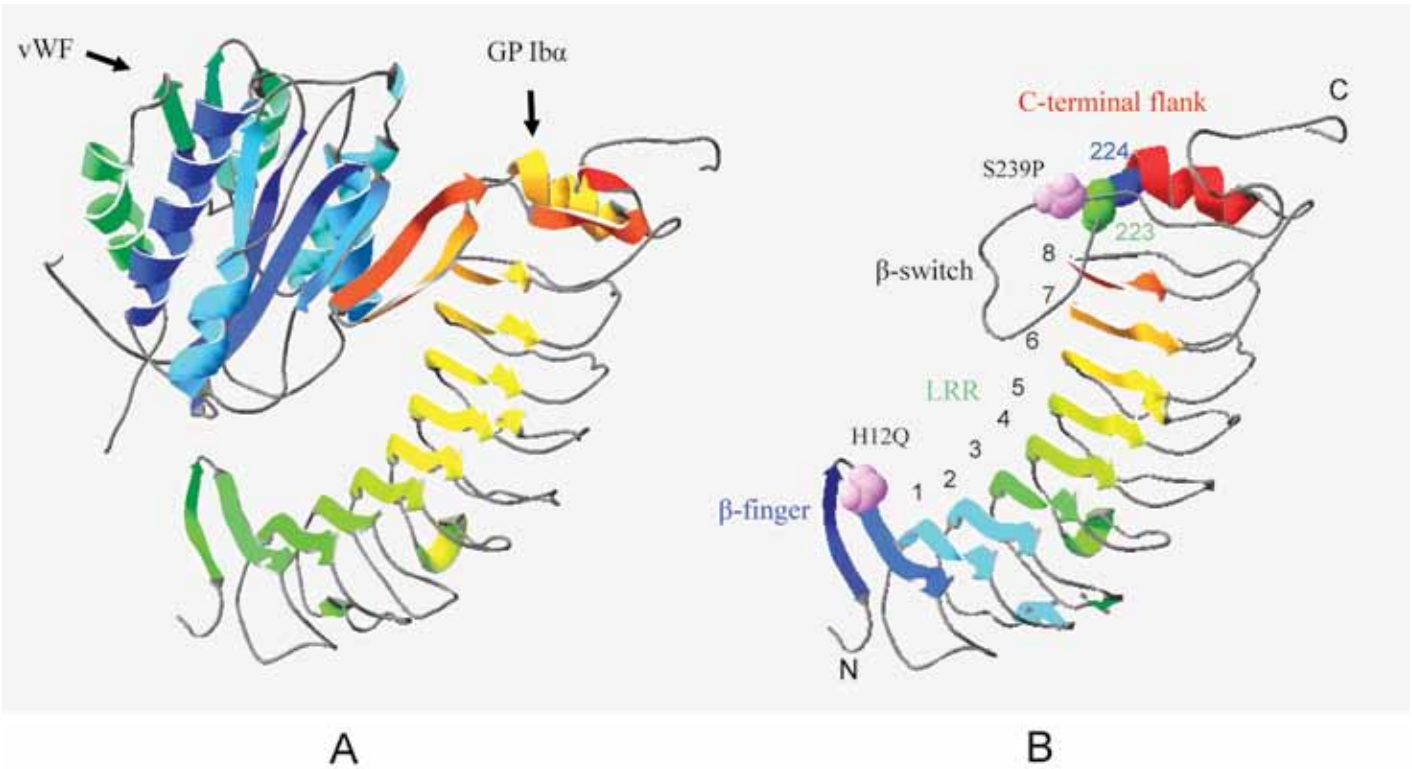

Figure 3. 3D modeling of porcine GPIbα. (A) 1M10 (Crystal structure of the complex of Glycoprotein Ib α and the von Willebrand Factor A1 domain) selected from PDB as primary template. (B) 3D models of porcine GPIbα. The picture shows the vWF binding domain of porcine GPIbα, followed (from N-terminal to C-terminal) by the β-finger motif, 8 leucine-rich repeats (LLR), and the C-terminal flanking region. The conformations of the porcine and human vWF binding sites of GPIbα were generally conserved. Among the specific binding sites for vWF, 2 residues were unique in porcine GPIbα compared with the human GPIbα (Gln12 and Pro241; shown as pink balls) . The residue corresponding to human Glu225 is missing from porcine GPIbα; on the model, this residue would have between the green and blue balls near the C-terminal region.

O-glycosylation sites, whereas the human homolog contained 5 O-glycosylation sites.

Comparison of the vWF binding sites. The N-terminal 290 residues of GPIbα contain the binding site for vWF; therefore, we compared the sequences of the major vWF binding sites of the porcine GPIbα and human proteins within the leucine-rich repeat domain (amino acids 0 through 267). The GPIbα residues that directly contact human vWF-A1 are located within the N-terminal flank (Ser11, His12, Glu14 and Asn16), the LRR domain (His37, Glu128, Lys152, Asp175, Thr176, and Phe199), and the C-terminal flank (Glu225, Asn226, Tyr228 and Ser241).[2] The alignment results revealed that 3 residues differ between the porcine and human vWF binding sites (Figure 2).

Comparison of the PEST domains. The PEST domain is a polypeptide sequence that is enriched in proline, glutamate, serine, and threonine[22] and that is proposed to expedite the degradation of proteins in which it is located.[20] Our analysis of the GPIbα sequence revealed a highly variant random-repeat PEST domain between the leucine-rich repeat domain and the transmembrane domain. Comparison of the deduced amino acid sequences of the porcine and human GPIbα PEST domains showed only 35.3% identity (Table 1).

3D modeling of porcine GPIbα. GPIbα anchors the GPIb–IX–V complex to the cytoskeleton and harbors the vWF-binding function.[13] We compared the conformations of various important functional sites in the porcine and human GPIbα proteins by using the knowledge-based comparative protein-modeling program SWISS-MODEL. The 3D structures used as the templates to generate the porcine GPIb models and to carry out the comparative study were obtained from the Protein Data Bank database. The 3D conformations of the porcine and human vWF binding sites of GPIbα were generally conserved (Figure 3). Despite marked variations in the nucleotide sequences, the backbone conformations were highly conserved between the 2 proteins.

Platelet aggregation. To compare the function of human and porcine GPIb–IX–V, we performed platelet aggregation tests by using platelet-rich plasma from healthy pigs and humans and the aggregation reagent ristocetin. The amount of ristocetin-induced aggregation differed greatly between human and porcine platelets (Figure 4). When treated with 0.9 mg/mL ristocetin, human platelets exhibited 76.2% aggregation on average, whereas porcine platelet-rich plasma showed 5.6% aggregation. Even at higher ristocetin doses of 1.8 mg/mL and 3.6 mg/mL, the aggregation rates were only 9.0% and 12.7%, respectively.

Discussion

We cloned the porcine GPIbα gene and compared the functional sites of the corresponding protein with those of the human protein. In addition, we evaluated the ristocetin-induced aggregation of porcine platelets. Our results suggest that the interaction between ristocetin, vWF, and GPIbα differs between pigs and humans, thereby informing our knowledge of platelets in

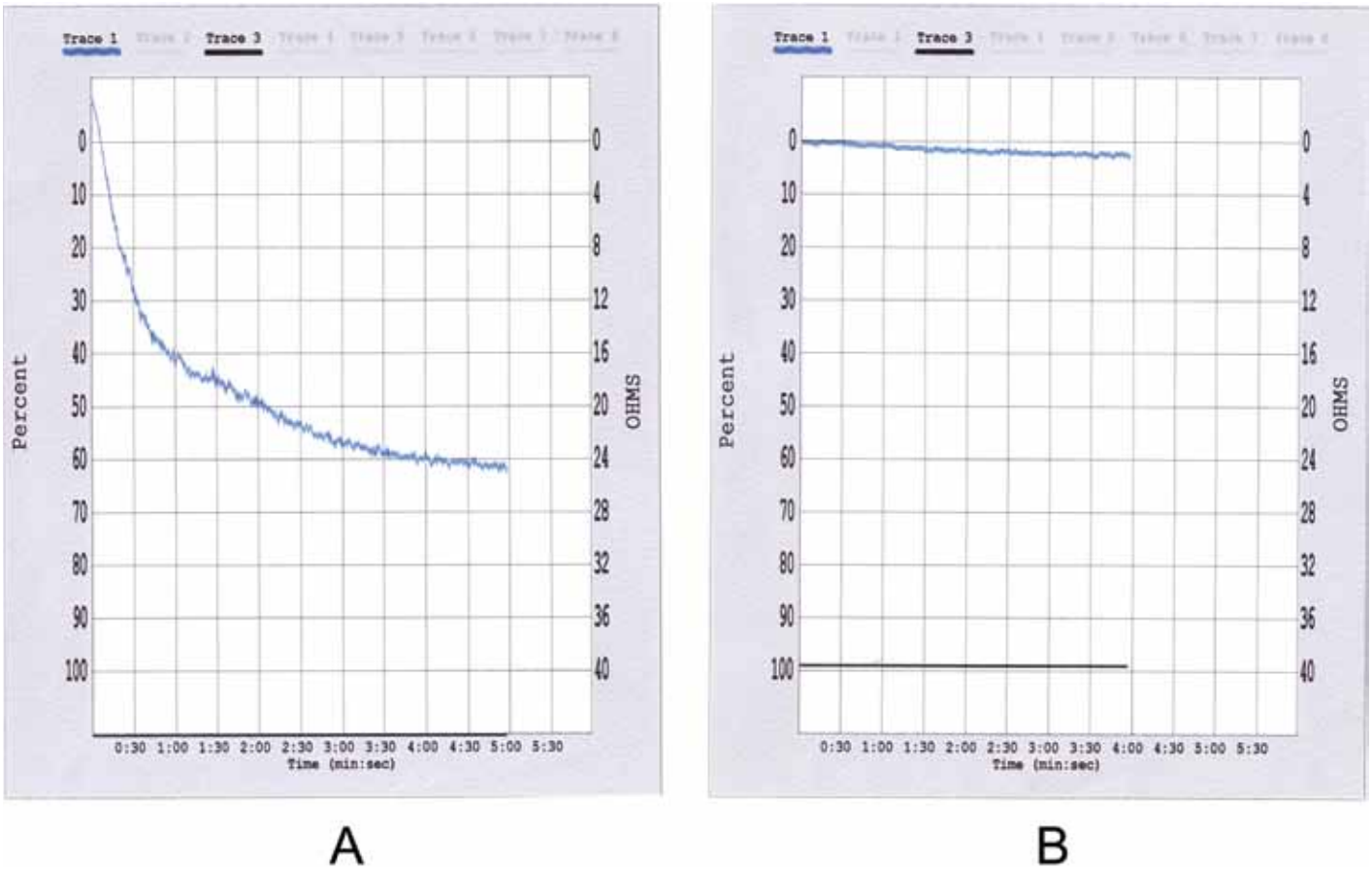

Figure 4. Representative images of ristocetin-induced platelet aggregation. (A) Normal human platelet aggregation in response to ristocetin (0.9 mg/mL) is shown. (B) Compared with human platelets, porcine platelets have decreased levels of aggregation even after treatment with 1.8 mg/mL ristocetin.

other species. When using pigs as models in coagulation studies, investigators should pay close attention to potential differences between pigs and humans.

Pigs have been used widely in biomedical studies for decades. However, relatively little information about the platelet physiology of pigs compared with humans has been reported. Our early studies revealed important differences in coagulation between pigs and humans.[5] Compared with humans, pigs have slightly longer prothrombin times and activated partial thromboplastin times, whereas the activities of coagulation factors VII and X in pigs are greater than those in healthy humans.[4,5] In addition, thromboelastography has shown that, compared with humans, pigs are in a hypercoagulable state.[5] In light of these coagulation parameters, we proposed that differences in platelet function may exist between pigs and humans. Pig-to-human (or nonhuman primate) xenotransplantation studies have confirmed that porcine vWF binds to human (or nonhuman primate) GPIbα on quiescent platelets, leading to platelet aggregation even in the absence of shear stress.[8] In vitro experiments also showed that pig vWF agglutinated human platelets with or without ristocetin, whereas pig platelets did not respond to ristocetin-activated human vWF.[34] These results suggest distinct vWF–GPIbα interaction patterns between pigs and human.

Recent studies have determined that the primary contact sites of human vWF on GPIbα are located in leucine-rich repeat domains 5 through 8 and in the C-terminal flank of GPIbα,[13] which interact with helix α3, loop α3β4, and strand β3 of the vWF A1 domain. The second and smaller contact site is formed by the N-terminal β finger and the first leucine-rich repeat domain of GPIbα; this contact site interacts with loops α1β2, α2β3, and α3β4 on the bottom face of the vWF A1 domain.[13] The specific vWF binding sites of human GPIbα were determined to be the N-terminal flanking region (Ser11, His12, Glu14, Asn16), the leucine-rich repeat domain (His37, Glu128, Lys152, Asp175, Thr176, Phe199), and the C-terminal flanking region (Glu225, Asn226, Tyr228, Ser241).[19] When comparing these major vWF binding sites of pig GPIbα with those of the human protein, we found 2 unique binding residues (Gln12, Pro241) and a residue (corresponding to human Glu225) is missing from porcine GPIbα. In addition to these 3 sites, we noted variations in the porcine β-switch (Val230Glu, Gln232Glu, and Ala238Val) that may alter the affinity of GPIbα for vWF.[8] These genetic changes in the vWF binding site of porcine GPIbα suggested to us that vWF-induced platelet aggregation may differ between pigs and humans, and this possibility warrants further investigation.

Ristocetin is an antibiotic obtained from *Amycolatopsis lurida* that causes platelet aggregation.[12] It is now used as a platelet activation agonist in clinical laboratories and as a reagent for in vitro tests of platelet function.[18,32] In the absence of high shear forces, ristocetin can be used to promote the in vitro binding of human

vWF to its platelet receptor GPIbα.[12,7] In the current study, low doses of ristocetin successfully induced normal human platelet aggregation; however, this compound failed to induce porcine platelet aggregation, even at a relatively high dose. This result suggests that the interaction between ristocetin and vWF–GPIbα differs between the 2 species, but the mechanism underlying this difference is unclear. The treatment of the PRP with ristocetin increases the affinity of vWF for the GPIb–IX–V complex. Ristocetin is proposed to binds vWF and alter the conformation of the A1 domain or to bridge the interaction between vWF and GPIbα.[16]

Although the molecular mechanisms of ristocetin-induced platelet aggregation are not yet fully defined, the regions involved in vWF–ristocetin–GPIbα interactions are located at amino acids 1237 through 1251 and 1457 through 1471 of vWF and at one or more sites in GPIbα.[10] Therefore, we speculate that the different results of the ristocetin-induced platelet aggregation tests between human and porcine may be, in part, due to species-specific aspects of the structure of vWF. When we analyzed these 2 regions of human and porcine vWF, we found that 8 of the 15 residues comprising the site at amino acids 1237 through 1251were the same between pigs and humans and that the porcine vWF protein had a notable 4-residue deletion in this region. In addition, 11 of the 15 amino acids comprising the interaction site at amino acids 1457 through 1471 of vWF were identical between humans and pigs.[25] This lack of complete homogeneity may lead to a decreased interaction among ristocetin, porcine vWF, and GPIbα, accounting for ristocetin's lack of effect on porcine platelets. Because little is known about the ristocetin-binding site on GPIbα, we were unable to assess whether differences were present between the human and porcine GPIbα proteins that might contribute to the poor response of porcine platelets to ristocetin. In addition, our genetic analysis of the vWF binding sites of porcine GPIbα revealed disparities between the GPIbα proteins of the 2 species that may lead to different vWF binding affinities, which also may contribute to the low ristocetin-induced aggregation of porcine platelets.

The PEST sequence between the N-terminal leucine-rich repeat and the C-terminal transmembrane domain showed the most noteworthy difference between pigs and humans. The functions of this PEST sequence have not yet been defined, but the PEST sequence is hypothesized to act as a signal peptide for protein degradation.[20] We suspect that PEST polymorphisms will be reflected in differences in the molecular weight and conformation of the total protein. The precise roles of the PEST domain merit further study.

Acknowledgments

This research was support by National Natural Science Foundation of China (grant no. 30930088) and the National Basic Research Program of China (grant no. 2009CB522401).

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