Enzymatic Amplification of Platelet-specific Messenger RNA Using the Polymerase Chain Reaction

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Abstract

Human platelets are derived from megakaryocytes as anucleate cells, and thus contain only vestigial amounts of RNA capable of being transcribed into protein. This has greatly hampered efforts to study directly platelet-specific gene products and their associated polymorphisms. In this report, we describe direct amplification, using the polymerase chain reaction, of platelet-derived mRNA in amounts sufficient to permit detailed analysis, such as restriction mapping and nucleotide sequencing. The ability to generate large amounts of cDNA from platelet-specific mRNA sequences should make possible direct molecular characterization of normal platelet proteins, and facilitate the investigation of a wide variety of inherited platelet disorders.

Introduction

Human platelets contain more than 40 identifiable membrane protein constituents, many of which have yet to be characterized. Since platelets are derived from megakaryocytes as nonnucleated cells, it is generally believed that they possess only vestigial amounts of protein-synthetic capability, although specific protein biosynthesis in platelets has been demonstrated (1–3). A primary deterrent to investigating the primary structure of platelet-specific gene products has therefore been the inability to obtain sufficient platelet mRNA to produce cDNA libraries. A number of nucleated cell types have recently been shown to synthesize proteins that are biochemically and immunologically related to their platelet counterparts (for a review, see reference 4), and cDNA libraries constructed from these cells have been used to obtain the nucleotide sequences of several platelet protein analogues (5–8). Since these sequences are deduced from cDNA clones derived from either transformed cell lines or cells of nonmegakaryocytic lineage, the possibility remains that the protein sequence of the actual platelet counterpart differs somewhat from that of the analogue found in the nucleated cell from which the sequence was derived.

It would be desirable to be able to study platelet-specific gene sequences for a number of other reasons. Platelets express a number of polymorphic epitopes on their surface that play an important role in the etiology of alloimmune platelet disorders (9, 10), and the molecular definition of alloantigenic determinants would contribute to our understanding of the morphological features of platelet antigens that are responsible for eliciting an alloimmune response. These and other interesting platelet-related polymorphisms are often present in low frequency in the human population, making it nearly impossible to predict or regulate the phenotypic makeup of a nonplatelet cDNA library. A preliminary report (11) suggests that platelet mRNA can be prepared from individuals with myoproliferative disorders in sufficient quantities to permit construction of a platelet-specific cDNA library, but it too would be limited in the number of polymorphic phenotypes represented.

Recently, a method to enzymatically amplify short segments of DNA has been developed (12–16). Known as the polymerase chain reaction (PcR),[1] this technique uses two oligonucleotide primers to direct the repeated synthesis of DNA bounded by their respective complementary sequences. Since PcR has been reported to be capable of amplifying specific DNA sequences $> 10^5$ times, we decided to explore using this method in the amplification of the vestigial amounts of mRNA present in human platelets. In this report, we demonstrate that the RNA that can be derived from the platelets present in 50 ml of whole blood from normal individuals is sufficient to produce microgram quantities of platelet-specific cDNA. This DNA is readily amenable to further detailed analysis, including restriction mapping, subcloning, and nucleotide sequencing.

Methods

Materials. Taq DNA polymerase isolated from *Thermus aquaticus* was purchased from Perkin-Elmer Corp., Instrument Div., Norwalk, CT. PGE_1, Sarkosyl, and Antifoam A were obtained from Sigma Chemical Co., St. Louis, MO. Agarose used for preparative electrophoresis was GTG-grade supplied by FMC Corp. Bioproducts, Rockland, ME. Placental RNAse inhibitor (RNAsin) was purchased from Promega Biotec, Madison, WI. Guanidinium isothiocyanate and cesium

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1. *Abbreviations used in this paper:* GP, glycoprotein; PcR, polymerase chain reaction; PRP, platelet-rich plasma.

chloride were purchased from Bethesda Research Laboratories, Gaithersburg, MD. Southern blot hybridization was performed after transfer to Genescreen Plus nylon membranes (Dupont Co., Diagnostic and BioResearch Systems, Wilmington, DE). All other chemicals used were of reagent grade.

Isolation of platelet RNA. Human platelets were isolated from whole blood as previously described (17). Briefly, 50 ml of venous blood collected from healthy volunteer donors was anticoagulated with 7 ml of acid citrate dextrose (National Institutes of Health, Formula A), and PGE_1 was added to a final concentration of 50 nmol/liter. Platelet-rich plasma (PRP) was prepared by differential centrifugation, and the platelets present in the upper half of the PRP were harvested and washed an additional two times in wash buffer (100 mmol/liter NaCl, 8.5 mmol/liter Tris, 8.5 mmol/liter glucose, and 1 mmol/liter Na_2EDTA, pH 7.4). By carefully avoiding the lower half of the PRP, we were consistently able to achieve a platelet/leukocyte ratio of at least 3000:1. The final platelet pellet was solubilized in 3.3 ml of buffer composed of 4 mol/liter guanidinium isothiocyanate, 5 mmol/liter sodium citrate, 0.1 mol/liter 2-mercaptoethanol, 0.5% Sarkosyl, 0.1% Antifoam A, and 40 U/ml placental RNAse inhibitor, layered onto a 1.2-ml cushion of 5.7 mol/liter CsCl, and centrifuged at 20°C in a Ti50.1 rotor at 35,000 rpm for 16 h (Beckman Instruments, Inc. Fullerton, CA). The nearly invisible RNA pellet was resuspended in buffer made up of 10 mmol/liter Tris, 5 mmol/liter EDTA, and 1% SDS, pH 7.4), extracted with chloroform/butanol, and ethanol precipitated essentially as described by Maniatis et al. (18). The resulting purified total platelet RNA fraction recovered using this technique was <1 μg per individual, and was used in all subsequent steps without further fractionation.

Oligonucleotides. Based upon the published nucleotide sequence of platelet membrane glycoprotein (GP) IIIa (5), two oligonucleotide primers were constructed such that they would be ~ 1 kb apart in the mRNA sequence. All oligonucleotides used in this study were constructed by The Blood Center of Southeastern Wisconsin Molecular Biology Core Laboratory (Milwaukee, WI) with a Gene Assembler (Pharmacia Fine Chemicals, Piscataway, NJ) using standard β-cyanoethyl phosphoramidite chemistry. Oligonucleotides were purified by preparative gel electrophoresis on sequencing gels followed by Sephadex G-25 (Pharmacia Fine Chemicals) chromatography. The oligo PcR-1 corresponded to the RNA strand from nucleotides 1,116–1,138 of the published sequence (5), whereas oligo PcR-2 corresponded to the antisense strand at nucleotides 2,201–2,177. The sequences of these primers are shown in Table I, with their locations relative to the coding sequence of GPIIIa cDNA illustrated in Fig. 1. The region bounded by these two oligonucleotides, while only 1,085 bases apart in the mRNA sequence, is known to span > 5 kb of genomic DNA, encompassing no less than three introns (L. Fitzgerald, Gladstone Foundation Laboratories, personal communication). The oligonucleotide primers correspond to unique sequences present in GPIIIa that are not present in related members of the integrin family of cytoadhesive glycoprotein receptors such as the leukocyte very late activation (VLA) antigens, LFA-1, or the fibronectin receptor (4–6). The specificity of each oligonucleotide was verified by Southern blot hybridization to DNA inserts of known identity (see below).

Amplification of the platelet GPIIIa RNA sequence. Total platelet RNA from each individual was resuspended in 28.7 μl of diethylpyrocarbonate-treated, sterile water in a sterile, siliconized, diethylpyrocarbonate-treated 0.5-ml polypropylene tube. cDNA synthesis was performed directly in this tube using cloned M-MLV reverse transcriptase and a cDNA synthesis kit (Bethesda Research Laboratories) according to the manufacturer's directions, except that the oligonucleotide primer PcR-2 was substituted for Oligo $(dT)_{12-18}$ to direct only GPIIIa-specific first strand synthesis. The first strand reaction mix contained 50 mmol/liter Tris pH 8.3, 75 mmol/liter KCl, 3 mmol/liter $MgCl_2$, 10 mmol/liter DTT, 0.5 mmol/liter each of dATP, dGTP, dCTP, and dTTP, 1.0 μmol/liter PcR-2 oligonucleotide, and 500 U M-MLV reverse transcriptase, in a final volume of 50 μl. cDNA synthesis was carried out at 37°C for 60 min, at which time the reaction was stopped by chilling to 0°C. The first strand reaction mix was diluted with an equal volume of PcR dilution buffer (25 mmol/liter KCl, 1 μmol/liter PcR2 oligonucleotide, 2 μmol/liter PcR-1 oligonucleotide, and 0.02% gelatin) and heated to 94°C for 2 min to separate the strands. Samples were allowed to cool to 37°C, at which time 1 μl (5 U) of Taq polymerase was added to initiate the polymerase chain reaction. Amplification was performed for 30 cycles, with 10-min primer extensions at 72°C, denaturation for 1 min 20 s at 94°C, and primer annealings for 3 min 20 s at 37°C. An additional 2.5 U of Taq polymerase was added after cycles 10 and 20. After the PcR, the amplified samples were stored at −20°C.

Analysis of PcR reaction products. 10–15 μl of each 100-μl PcR reaction was analyzed on a 1% agarose gel, followed by staining with ethidium bromide. Positive identification of the resulting DNA bands as authentic derivatives of the mRNA encoding GPIIIa was obtained by Southern blot analysis, using a cloned GPIIIa insert that runs from base 45 of the published sequence (5) to the internal Eco R1 site at base 2,274. This GPIIIa clone, termed 52, was obtained from a λgt11 expression library (kindly provided by Dr. C.-J. Edgell, University of North Carolina, Chapel Hill, NC) made from the permanent endothelial cell/lung carcinoma hybrid cell line, EA·hy 926, (19). The identity of clone 52 was verified by both restriction mapping and comparison of its nucleic acid sequence (not shown) with that of the published sequence for GPIIIa. Southern blots were hybridized at 68°C for 16 h with a ^{32}P-labeled nicktranslated ptZ18r plasmid (Pharmacia Fine Chemicals) that contained the clone 52 insert, washed at high stringency (0.1× SSC, 68°C), and exposed to Kodak X-AR film for 15–30 min.

To further characterize the PcR reaction products, amplified DNA bands of the expected size were recovered from agarose gels using an electroeluter (model UEA; International Biotechnologies Inc., New Haven, CT) and subjected to limited restriction map analysis. Selected restriction fragments were subcloned into the plasmid vector pGEM-7Zf (Promega Biotec) and subjected to nucleotide sequence analysis.

Results

Amplification of the GPIIIa mRNA. As an example of the utility of PcR in amplifying platelet-specific mRNAs, we chose to perform our initial studies using the well-characterized GPIIIa molecule. The amplification strategy to produce a platelet-specific cDNA is depicted in Fig. 1. To facilitate analysis of the PcR reaction products, two oligonucleotide primers, PcR-1 and PcR-2, were constructed such that they would: (*a*) complement sequences specific for GPIIIa, and (*b*) flank a

Table I. Oligonucleotides Corresponding to Platelet-specific Sequences Used in cDNA Synthesis and PcR Amplification

Primer	Sequence location in GPIIIa	DNA strand	Sequence (5′ – – –> 3′)
PcR-1	1,116–1,138	RNA	GTCCTCCAGCTCATTGTTGATGC
PcR-2	2,201–2,177	Anti-sense	CATCACTGAGAGCAGGACCACCAGG

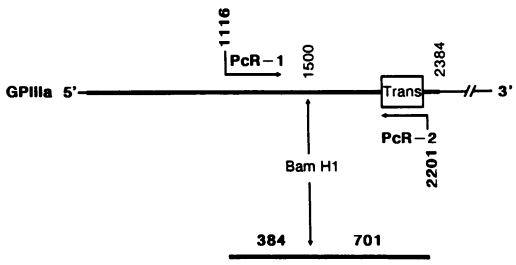

Figure 1. Diagramatic representation of the GPIIIa mRNA molecule. The locations of the two PcR oligonucleotide primers, the region encoding the putative transmembrane domain (Trans), the unique Bam H1 restriction site, and the predicted 1-kb section of the amplified GPIIIa cDNA (*bottom*) are illustrated.

unique Bam H1 restriction endonuclease site. The relatively large 1,000-bp distance between the two oligonucleotides was chosen to provide a stringent test of the specificity and overall utility of PcR in amplifying the minute amounts of vestigial RNA present in human platelets. As shown in Fig. 2, large amounts of a 1-kb cDNA were produced during the PcR (lane *A*). Also evident were lesser amounts of smaller sized cDNA's. Most of these probably represent sites of nonspecific priming on other platelet RNAs, although a few bands may represent smaller reaction products related to the GPIIIa mRNA (discussed below).

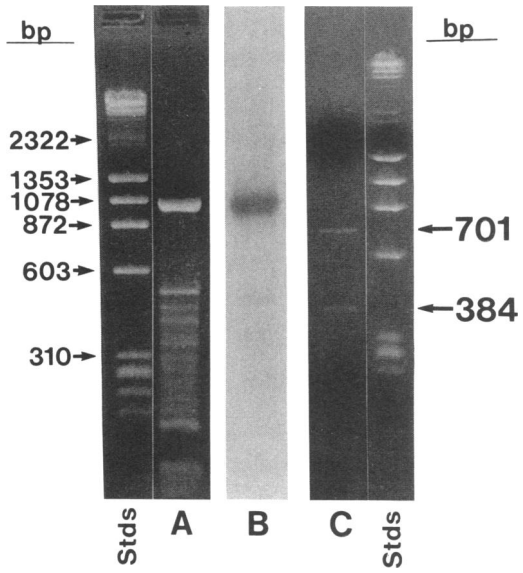

Figure 2. Identification and characterization of the cDNA molecule amplified from platelet mRNA. Total platelet RNA derived from 50 ml of blood was enzymatically amplified in the presence of the oligonucleotide primers, PcR-1 and PcR-2, using Taq polymerase for 30 cycles in a total reaction volume of 100 μl. After PcR, 15 μl of the reaction mixture was analyzed on a 1% agarose gel, and the resulting bands visualized by ethidium bromide staining (lane *A*). Lane *B* is an autoradiograph of a Southern blot of the same gel that had been hybridized with a nick-translated plasmid containing a GPIIIa insert. The remaining 85 μl of the PcR reaction mix was subjected to preparative agarose gel electrophoresis, and the 1-kb band excised and recovered by electroelution. 20% of this material was digested with Bam H1, resulting in restriction fragments of the expected sizes (lane *C*).

Characterization of the 1-kb cDNA produced during PcR. To identify the major band (Fig. 2, lane *A*) as a GPIIIa cDNA, the same ethidium bromide-stained gel was further analyzed by Southern blot hybridization. As shown in Fig. 2, lane *B*, the major band formed during PcR hybridized strongly with the GPIIIa-specific probe. Several, but not all, of the minor PcR reaction products also hybridized with the probe, suggesting that they may represent incomplete or partially degraded GPIIIa cDNAs. The remaining PcR bands did not show specific hybridization, and may have formed as a result of the relatively low temperature (37°C) used during the primer annealing step of the PcR.

To provide further evidence that the major cDNA produced during PcR was an authentic amplified copy of the region of GPIIIa bounded by the oligonucleotides PcR-1 and PcR-2, the band at 1 kb that had hybridized with the known GPIIIa cDNA insert was excised from a preparative agarose gel, purified by electroelution, and digested with the restriction endonuclease Bam H1. Two discrete fragments, migrating at 384 and 701 bp, resulted from this digestion (Fig. 2, lane *C*). These bands correspond exactly to the predicted sizes (see Fig. 1), and together with the hybridization data, positively identify the major product of the PcR reaction as the expected segment of cDNA encoding platelet GPIIIa. Nucleotide sequence analysis of the two restriction fragments (not shown) confirmed these results.

Discussion

We have described a new approach for studying directly platelet mRNA sequences that encode platelet-specific gene products. Our strategy is based on the use of the PcR, which has been shown to be capable of enzymatically amplifying short segments of DNA (12–16). To adapt the amplification technique to the study of platelet-specific proteins, we had to convert platelet-specific mRNA into cDNA before performing PcR. The amount of platelet RNA that can be recovered from 50 ml of blood from normal individuals is exceedingly small, thus both RNA and cDNA preparation were performed using samples that could not generally be visualized. Using two oligonucleotide primers that flank a 1-kb region present in platelet GPIIIa, however, we were able to synthesize, in vitro, microgram amounts of a specifically targeted region of the GPIIIa cDNA molecule. That the resulting amplified cDNA represented an authentic copy of platelet GPIIIa cDNA was demonstrated by hybridization to a well-characterized specific probe, and by the generation of restriction fragments diagnostic for the specific region of the GPIIIa cDNA under investigation. Subsequent nucleotide sequence analysis of the amplified segment confirmed these results.

The ability to amplify specific sequences 10^5–10^6 times affords a level of sensitivity heretofore unavailable for investigating the presence of small quantities of distinct mRNAs in individual cell types. This capability will undoubtedly lead to the discovery that proteins once thought to be cell- or tissue-specific are present in previously undetectable quantities in other cell types. For example, there is presently no consensus, based upon extensive immunochemical analysis from a number of laboratories, as to whether peripheral blood monocytes actually synthesize small amounts of GPIIIa, or merely appear to express it on their surface as a result of contamination with

adherent platelets or platelet fragments (4). Northern blot analysis of monocyte mRNA, however, failed to demonstrate specific messages encoding either GPIIb or GPIIIa (8). Therefore, if monocytes contain GPIIb/GPIIIa mRNAs, they are present at levels below those which are detectable by northern blot analysis. Using the techniques presented here, questions such as these can now be directly addressed.

Current methods for purifying platelets by differential centrifugation are incapable of yielding absolutely leukocyte-free preparations. Although there are currently no convincing data that GPIIIa is expressed in any leukocyte subpopulation, it cannot yet be excluded that our platelet RNA preparation contains some proportion of monocyte, neutrophil, and lymphocyte RNA, and that a small percentage of the amplified GPIIIa cDNA could be derived from leukocyte mRNA. That the majority of the RNA is of platelet origin, however, is supported by the fact that our laboratory has also been successful in amplifying from our platelet RNA preparations vWf cDNA, which encodes a protein found exclusively in endothelial cells and platelets (20) (Friedman, K., P. Newman, J. Rayner, and R. Montgomery, unpublished observations).

The discovery that platelet-specific mRNA sequences can be amplified using PcR is applicable to a wide variety of topics related to platelet morphology and physiology. For example, the mere fact that platelets contain mRNA sequences that can be amplified strongly supports the evidence obtained by others (1–3) that platelets retain the ability to synthesize small amounts of protein. It would be interesting to see if all platelets have this capability, or whether the presence of RNA is restricted to young platelets recently released from bone marrow megakaryocytes. If the latter is the case, PcR might be used to investigate questions of platelet aging, such as the relationship between platelet size, age, and density. Another anticipated application of PcR technology to the study of platelet pathophysiology might be genotypic analysis and diagnosis of Glanzmann's thrombasthenia, including defining the molecular basis of several thrombasthenic variants that have been reported (21, 22). Similarly, since there is some question as to the biosynthetic source of several platelet α-granule proteins, i.e., that platelets may be capable of acquiring plasma proteins through an endocytotic pathway (2, 23, 24), PcR could also be used to assess the ability of platelets, or indirectly megakaryocytes, to specifically synthesize individual platelet components.

Of particular interest to us is the potential applicability of PcR towards defining platelet-specific polymorphisms that result in allo- and auto-immune thrombocytopenias. PcR would appear to be the method of choice for analyzing relatively infrequent polymorphic DNA sequences, since it enables one to analyze specific sequence variations from a number of individuals without the need for cloning or constructing multiple cDNA libraries. The relative simplicity of the DNA amplification technique, together with the ability to now analyze platelet-specific sequences, should make possible the direct molecular characterization of normal platelet proteins and facilitate the investigation of a variety of inherited platelet disorders.

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References

1. Plow, E. F. 1979. Extracellular factors influencing the in vitro protein synthesis of platelets. *Thromb. Haemostasis.* 42:666–678.

2. Kieffer N., J. Guichard, J.-P. Farcet, W. Vainchenker, and J. Breton-Gorius. 1987. Biosynthesis of major platelet proteins in human platelets. *Eur. J. Biochem.* 164:189–195.

3. Belloc, F., E. Heilmann, R. Combrie, M. R. Boisseau, and A. T. Nurden. 1987. Protein synthesis and storage in human platelets: a defective storage of fibrinogen in platelets in Glanzmann's thrombasthenia. *Biochim. Biophys. Acta.* 925:218–225.

4. Newman, P. J. 1989. Phylogeny and tissue distribution of platelet antigens. *In* Platelet Immunology. T. J. Kunicki and J. N. George, editors. J. B. Lippincott, Co., Philadelphia, PA. In press.

5. Fitzgerald, L. A., B. Steiner, S. C. Rall, Jr., S.-S. Lo, and D. R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet glycoprotein IIIa and similarity to integrin. *J. Biol. Chem.* 262:3936–3939.

6. Poncz, M., R. Eisman, R. Hiedenreich, S. M. Silver, G. Vilaire, S. Surrey, E. Schwartz, and J. S. Bennett. 1987. Structure of the platelet membrane glycoprotein IIb. Homology to the α subunits of the vitronectin and fibronectin membrane receptors. *J. Biol. Chem.* 262:8476–8482.

7. Loftus, J. C., E. F. Plow, A. L. Frelinger III, S. E. D'Souza, D. Dixon, J. Lacy, J. Sorge, and M. H. Ginsberg. 1987. Molecular cloning and chemical synthesis of a region of platelet glycoprotein IIb involved in adhesive function. *Proc. Natl. Acad. Sci. USA.* 84:7114–7118.

8. Bray, P. F., J.-P. Rosa, G. I. Johnston, D. T. Shiu, R. G. Cook, C. Lau, Y. W. Kan, R. P. McEver, and M. A. Shuman. 1987. Platelet glycoprotein IIb. Chromosomal localization and tissue expression. *J. Clin. Invest.* 80:1812–1817.

9. Kunicki, T. J., and P. J. Newman. 1986. The biochemistry of platelet-specific antigens. *In* Current Studies in Hematology and Blood Transfusion. A. Hassig, editor. S. Karger, Basel, Switzerland. 18–32.

10. Aster, R. H. 1984. Clinical significance of platelet-specific antigens and antibodies. *In* Advances in Immunobiology and Bone Marrow Transplantation. J. McCullough and G. Sandler, editors. Allan R. Liss, Co., New York, 103–118.

11. Hickstein, D. D., M. J. Hickey, D. W. Chung, J. P. Lopez, and G. J. Roth. 1987. Isolation of Poly A (+) RNA from human neutrophils and platelets. *Blood.* 70:89*a*. (Abstr.)

12. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β-globin gene sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science (Wash. DC).* 230:1350–1354.

13. Scharf, S., G. T. Horn, and H. A. Erlich. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science (Wash. DC).* 233:1076–1078.

14. Kogan, S. C., M. Doherty, and J. Gitschier. 1987. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. Application to Hemophilia A. *N. Engl. J. Med.* 317:985–990.

15. Wong, C., C. E. Dowling, R. K. Saiki, R. G. Higuchi, H. A. Erlich, and H. H. Kazazian Jr. 1987. Characterization of β-thalassaemia mutations using direct genomic sequencing of amplified single copy DNA. *Nature (Lond.).* 330:384–386.

16. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC)*. 239:487–494.

17. Newman, P. J., R. P. McEver, M. P. Doers, and T. J. Kunicki. 1987. Synergistic action of two murine monoclonal antibodies the inhibit ADP-induced platelet aggregation without blocking fibrinogen binding. *Blood.* 69:668–676.

18. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

19. Edgell, C.-J. S., C. C. McDonald, and J. B. Graham. 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. USA.* 80:3734–3737.

20. Kawai, Y., and R. R. Montgomery. 1987. Endothelial cell processing of von Willebrand proteins. *Ann. NY Acad. Sci.* 509:60–70.

21. Ginsberg, M. H., A. Lightsey, T. J. Kunicki, A. Kaufmann, G. Marguerie, and E. F. Plow. 1986. Divalent cation regulation of the surface orientation of platelet membrane glycoprotein IIb. Correlation with fibrinogen binding function and definition of a novel variant of Glanzmann's thrombasthenia. *J. Clin. Invest.* 78:1103–1111.

22. Nurden, A. T., J.-P. Rosa, D. Fournier, C. Legrand, D. Didry, A. Parquet, and D. Pidard. 1987. A variant of Glanzmann's thrombasthenia with abnormal glycoprotein IIb-IIIa complexes in the platelet membrane. *J. Clin. Invest.* 79:962–969.

23. George, J. N., S. Saucerman, S. P. Levine, L. K. Kneiriem, and D. F. Bainton. 1985. Immunoglobulin G is a platelet alpha granule-secreted protein. *J. Clin. Invest.* 76:2020–2025.

24. Handagama, P. J., J. N. George, M. A. Shuman, R. P. McEver, and D. F. Bainton. 1987. Incorporation of circulating protein into megakaryocyte and platelet granules. *Proc. Natl. Acad. Sci. USA.* 84:861–865.