# ORIGINAL ARTICLES

# Calcium-Diacylglycerol Guanine Nucleotide Exchange Factor I gene mutations associated with loss of function in canine platelets

**MARY K. BOUDREAUX, JAMES L. CATALFAMO, and MARION KLOK**

AUBURN, ALA; ITHACA, NY; AND OUDESCHANS, THE NETHERLANDS

**Calcium-Diacylglycerol Guanine Nucleotide Exchange Factor I (CalDAG-GEFI) has been implicated in platelet aggregation signaling in CalDAG-GEFI knockouts. Functional mutations were identified in the gene encoding for CalDAG-GEFI in 3 dog breeds. Affected dogs experienced epistaxis, gingival bleeding, and petechiation. Platelet number, von Willebrand factor, clot retraction, and coagulation screening assays were normal, whereas bleeding time tests were prolonged. Platelet aggregation and release responses to all agonists, except thrombin, were markedly impaired. Platelet membranes had normal concentrations of integrin alphaIIb-beta3; however, ADP-induced fibrinogen binding by activated platelets was markedly impaired. Forskolin-stimulated platelets exhibited a marked increase in intraplatelet cAMP associated with impaired phosphodiesterase (PDE) activity, whereas levels of extractable phosphoinositides were 1.5-fold to 2-fold higher in thrombin-stimulated affected platelets. DNA analysis of the CalDAG-GEFI gene in affected dogs documented the existence of 3 distinct mutations within portions of the CalDAG-GEFI gene encoding for structurally conserved regions within the catalytic domain of the protein. The mutations are predicted to result in either lack of synthesis, enhanced degradation, or marked impairment of protein function. The dysfunctional profile of canine platelets observed in mutant dogs putatively links CalDAG-GEFI and its target Rap1 or other Ras family member, for the first time, to a role in pathways that regulate cAMP PDE activity and thrombin-stimulated phosphoinositide anchoring or metabolism. The finding of distinct functional mutations in 3 dog breeds suggests that mutations in the CalDAG-GEFI gene may be implicated in similar defects in human patients with congenital platelet disorders having primary secretion defects of unknown etiology. (Translational Research 2007;150:81–92)**

**Abbreviations:** CalDAG-GEFI = Calcium-Diacylglycerol Guanine Nucleotide Exchange Factor I; CAP-1 = canine activated platelet-1; EDTA = ethylenediaminetetraacetic acid; GEF = guanine nucleotide exchange factor; GT = Glanzmann thrombasthenia; GTP = guanine-nucleotide-binding protein; PAF = platelet activating factor; PAR = protease activated receptor; PCR = polymerase chain reaction; PDE = phosphodiesterase; PKC = protein kinase C; PRP = platelet rich plasma; RIBS = receptor induced binding site; SCR = structurally conserved region; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; TRAP = thrombin receptor activation peptide

The platelet integrin alphaIIb-beta3, also known as the platelet glycoprotein complex IIb-IIIa, mediates platelet aggregation by binding the dimeric ligand fibrinogen. In unactivated platelets, the integrin is in a low-affinity/avidity state for the binding of fibrinogen. The conversion from a low-affinity to a high-affinity state is mediated by signal transduction proteins mobilized in response to agonists binding to

From the Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, Ala; the Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY; and the Voorstraat 2, NL-9696 XH Oudeschans, The Netherlands.

Submitted for publication December 8, 2006; revison submitted March 7, 2007; accepted for publication March 10, 2007.

Reprint requests: Mary K. Boudreaux, DVM, PhD, Department of Pathobiology, 166 Greene Hall, College of Veterinary Medicine, Auburn University, Auburn, Ala 36849-5519; e-mail: boudrmk@auburn.edu.

1931-5244/$ – see front matter

© 2007 Mosby, Inc. All rights reserved.

doi:10.1016/j.trsl.2007.03.006

specific receptors on the platelet surface; this sequence of events is termed "inside-out" signaling.[1,2] Examples of agonists that can induce "inside-out" signaling and thus change the affinity of the integrin for fibrinogen include ADP, collagen, thromboxane, and thrombin. Once fibrinogen has bound to alphaIIb-beta3, signal transduction events referred to as "outside-in" signaling occur, which increase the avidity of the integrin for fibrinogen and are accompanied by integrin receptor clustering. Rap1 is a Ras-related low-molecular-weight guanine-nucleotide-binding protein (GTPase) that is ubiquitously expressed in high levels in platelets, neutrophils, and brain.[3] Rap1 seems to play an important role in several cell processes, including cell proliferation and differentiation, platelet, neutrophil, and B-cell activation, induction of T-cell anergy, and the regulation of the respiratory burst in neutrophils.[3] Rap1 is activated by many different stimuli in different cell types and is thus a shared (common) component in signaling. Receptor-signaled generation of second messengers, including calcium, cAMP, and DAG can lead to the direct activation of Rap1-specific guanine nucleotide exchange factors (GEFs). Platelets contain high levels of Rap1b. In platelets, Rap1 is involved in integrin activation and is activated by the binding of GTP and inactivated by the hydrolysis of bound GTP to GDP. In quiescent platelets, Rap1b is primarily localized to the plasma membrane.[4] After activation, Rap1b relocalizes to the actin cytoskeleton.[5] Platelet agonists that stimulate fibrinogen binding to platelet integrin alphaIIb-beta3 also stimulate binding of GTP to Rap1b resulting in its rapid activation.[6,7] Most physiologic agonists (including ADP and collagen) that stimulate Rap1b activation do so through stimulation of Gi-coupled receptors.[8,9] Platelet adhesion mediated by binding of the platelet integrin alpha2-beta1 to collagen initiates outside-in signaling pathways that also result in activation of Rap1b.[10] Agonists that activate platelets through Gq-coupled receptors, including thromboxane, or through cross-linking of Fc$\gamma$RIIA, which is a tyrosine kinase-based pathway, rely on binding of secreted ADP, which in turn binds to the Gi-coupled ADP receptor P2Y12.[8] The importance of Rap1b for normal platelet function and hemostasis was demonstrated recently in a knockout mouse model. Rap1b null mice exhibited markedly impaired platelet function and experienced 85% embryonic and perinatal lethality primarily caused by hemorrhage-related events.[11] Calcium-Diacylglycerol Guanine Nucleotide Exchange Factor I (CalDAG-GEFI) is a GEF that activates Rap1b. Guanine nucleotide exchange factors promote nucleotide release from GTPases. GDP is not preferentially released over GTP; cellular concentrations of GTP are generally 10 times higher than concentrations of GDP; thus, GTP has a higher likelihood of rebinding to the GTPase than GDP.[12] The exchange of GTP for GDP promotes the activity of GTPases. CalDAG-GEFI knockout mice exhibit severely impaired platelet aggregation and release responses to most agonists, including ADP, collagen, thromboxane, and the calcium ionophore A23187.[13] CalDAG-GEFI effects are likely caused by affinity/avidity modulation of integrin alphaIIb-beta3 via activation of Rap1b.[14] It is possible, however, not all the CalDAG-GEFI effects on platelet function reported in the knockout mice may be linked to activation of Rap1b. Intrinsic platelet function disorders have been described in Basset hounds and Eskimo Spitz dogs.[15-17] A platelet defect has been recognized in Landseers, a European dog breed related to the Newfoundland breed; however, biochemical and functional descriptions of the disorder are lacking. Affected Basset hounds, Spitz dogs, and Landseers experience epistaxis, gingival bleeding, and petechiation on mucous membranes and skin. Platelet number, plasma von Willebrand factor concentration, and function and coagulation screening assays are normal, whereas bleeding time tests are prolonged. In contrast to Rap1b null mice, reduced litter sizes or high neonatal lethality have not been described with these disorders in dogs.

The platelet disorders in Basset hounds and Spitz dogs have been well characterized, and they are essentially similar at the functional level. Platelet aggregation responses to ADP, collagen, calcium ionophore A23187, and platelet activating factor (PAF) are severely impaired. In response to thrombin, their platelets exhibit a characteristic lag phase with normal maximal extent aggregation. Epinephrine enhances the sensitivity of thrombopathic platelets to ADP, but the aggregation response is still reduced compared with normal platelets. Thrombopathic Basset hound intraplatelet fibrinogen content and concentrations of membrane glycoproteins IIb and IIIa are normal. Platelets from affected Basset hounds and Spitz dogs support normal clot retraction. cDNA sequences and coding areas of genomic DNA for platelet glycoproteins IIb and IIIa obtained from affected Basset hounds and heterozygous Landseers are identical to sequences obtained for normal dogs except for the presence of benign polymorphisms (Boudreaux, unpublished findings). The similarity of the platelet function disorder described in affected dogs to that identified in CalDAG-GEFI knockout mice, combined with the lack of embryonic or neonatal lethality described in the CalDAG-GEFI mouse knockout, prompted the evaluation of the gene encoding CalDAG-GEFI in affected Basset hounds, in an affected Eskimo Spitz dog, and in an affected Landseer. Three distinct CalDAG-GEFI gene mutations were identified in each breed evaluated. All mutations

**Primers used to amplify cDNA segments:**

| | | | |
|---|---|---|---|
| Pre Exon 1f | GAGGCCCAGAGTGCAGCGTGA | forward | |
| Exon 6r | GTGATGACCAGGGCACGCTGA | reverse | 718 bp |
| Exon 6f | CTCATGGCTGCACCGTGGACA | forward | |
| Exon 11r | GTCCCCAAAGGCGCTGAGGTA | reverse | 846 bp |
| Exon 11f | GGGGATGGCCACATCTCACAG | forward | |
| UTRr | GCTTCCTGCTCTGGTCCAAGT | reverse | 565 bp |

**Primers used to amplify DNA segments:**

| | | | |
|---|---|---|---|
| Pre Exon 1f | GAGGCCCAGAGTGCAGCGTGA | forward | |
| Intron 2r | GCTCTAGGAGCGAAGCCCAAT | reverse | 981 bp |
| Intron 2f | CCCTGCAGTCCCAGTCCATGA | forward | |
| Intron 3r | GCCAGTCTGAAGCCTGAGCAA | reverse | 809 bp |
| Intron 2f end | GCCACCCAGGCATCCCAGTTT | forward | |
| Intron 4r | GGTGACAGGGAGCCAGAGGTT | reverse | 708 bp |
| Intron 4f | GCAAAGGGCTCATTGCCCTTG | forward | |
| Intron 5r | CCCTCCAGCTGCCCTTCATTG | reverse | 354 bp |
| Intron 5f | GCCTTTGGTCCAGGGTGGAGT | forward | |
| Intron 7r | CCCAGGGCTTGATGAGGTTCT | reverse | 706 bp |
| Intron 7f | GGGTGTTCCCTTGAGCTCATG | forward | |
| Intron 8r | GAGCCCAGGCTGGATCTCAGT | reverse | 645 bp |
| Intron 8f | GACAGTGGGCTCCTGAGGACA | forward | |
| Exon 11r | GTCCCCAAAGGCGCTGAGGTA | reverse | 909 bp |
| Intron 10f | GGAGAGCCACTCACGTCTGAG | forward | |
| Intron 11r | CCTCTGCCGAGATCATCTGGT | reverse | 334 bp |
| Intron 11af | CAGGAGTGGGGTGAGGATCTT | forward | |
| Intron 13r | GCTGCAAGGTACTTCGCTGCT | reverse | 679 bp |
| Intron 12f | CCGAAAGCACCAGGGTCAGTG | forward | |
| Intron 14r | CTCCCCTCACAGGCCAATACA | reverse | 728 bp |
| Intron 14f | GCATCGAGCCTTCCAGAAGTG | forward | |
| UTRr | GCTTCCTGCTCTGGTCCAAGT | reverse | 565 bp |

**Fig 1.** Primer sets used to amplify cDNA and DNA segments of the gene encoding CalDAG-GEFI. The length of the segments amplified are indicated following the primer sets (bp = base pairs).

were located in portions of the gene encoding the highly conserved catalytic unit. The changes are considered significant and would result in either lack of synthesis, enhanced degradation, or marked impairment of protein function. This article illustrates the effects of mutations in a gene encoding for a critically important GEF in platelets in 3 different dog breeds and the striking similarity of these effects to those observed in CalDAG-GEFI knockout mice.

## MATERIALS AND METHODS

This study was conducted in compliance with ethical guidelines for research involving the use of animals.

**cDNA synthesis.** Platelet rich plasma (PRP) was isolated from ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood using differential centrifugation. Prostaglandin $E_1$ was added to PRP samples at a final concentration of 3 $\mu$M before centrifugation of PRP to form pellets. Platelet pellets were resuspended in a small volume of autologous plasma and transferred to RNase-free tubes and centrifuged again. Residual plasma was removed from the pellets, and the pellets were frozen at −80°C until use for RNA isolation. Total RNA was isolated from platelet pellets using a commercially available kit (Micro to Midi Total RNA Purification Kit; Invitrogen, Carlsbad, Calif). cDNA synthesis was accomplished using a separate commercially available kit (iScript cDNA synthesis kit; BioRad, Hercules, Calif).

**Genomic DNA isolation.** Genomic DNA was harvested from EDTA-anticoagulated whole blood using a commercially available kit (QIAamp DNA Blood Mini Kit; Qiagen, Valencia, Calif).

**Polymerase chain reaction (PCR) analysis.** Primers for CalDAG-GEFI were designed based on sequence information found on GenBank (GI:73983733, PREDICTED: Canis familiaris sequence similar to RAS guanyl releasing protein 2 isoform 1, mRNA) and sequence within the dog genome located using mRNA sequence information (Fig 1). Primers for protease activated receptor (PAR) 1, PAR3, and PAR4 were designed based on human and mouse sequences located on GenBank. cDNA and DNA segments were initially amplified by PCR by using normal canine cDNA or DNA as a template. Amplification products were separated on agarose gels using electrophoresis. DNA was extracted from target bands using a commercially available kit (QIAquick Gel Extraction Kit; Qiagen). Extracted DNA was submitted for DNA sequencing in a laboratory equipped with an ABI 3100 Genetic Analyzer.

**Flow cytometry.** Citrated PRP (10 $\mu$L) was added to 100 $\mu$L of flow buffer (10mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, pH 7.4) in round-bottomed polystyrene tubes. Monoclonal antibodies were added directly to samples of diluted PRP. Monoclonal antibodies evaluated included Y2/51, an FITC-labeled antibody against human platelet glycoprotein IIIa (DAKO, Carpinteria, California) that recognizes canine IIIa, 2F9 a monoclonal antibody recognizing canine IIb (kind gift from Dr. S.A. Burstein, University of Oklahoma Health Sciences Center), and canine activated platelet-1 (CAP-1), a monoclonal antibody recognizing a receptor induced binding site (RIBS) on canine fibrinogen.[18] The FITC-labeled isotype control antibody was used for samples evaluating binding of Y2/51. CAP-1 binding was evaluated using a secondary, FITC-labeled antibody, which was also used without primary antibody in control experiments to detect nonspecific binding. CAP-1 binding was evaluated in nonactivated and activated PRP samples. Samples were fixed with 500 $\mu$L of 2% formalin and immediately evaluated by flow cytometry (Coulter Epics Elite, Hialeah, Fla).

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.** Human and canine blood was collected into ACD-A, and platelets were isolated essentially as described for phospholipid analysis.[19] After the second wash, platelets were resuspended to a final concentration of $3 \times 10^8$ platelets per 1.0 mL wash buffer and 2, 10-$\mu$L aliquots were removed for protein determination (BCA Protein Assay Reagent Kit; Pierce Biotechnology, Rockford, Ill). The remaining cell suspension was recentrifuged. The supernatants were discarded, and platelet pellets were stored frozen at −70°C until analysis. The frozen platelet pellets were lysed in hot (95°C) Laemelli SDS-PAGE sample buffer (BioRad) with vortex mixing and heated for 4 min at 95°C. Platelet proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels (Ready Gels, BioRad) with 40-$\mu$g protein loaded per lane and then electroblotted onto PVDF membranes (BioRad) using cooled Tris-glycine buffer (25 mM Tris, pH 8.3, 192 mM glycine). The PVDF membranes were then blocked overnight at 4–8°C using 5% (wt/vol) powdered milk in TBS-T (20 mM Tris HCL, pH 7.5, 500 mM NaCl, 0.1% (wt/vol) Tween-20). The blots were washed 3 times in TBS-T and probed for 1 h at 18–24°C using a purified mouse monoclonal antibody (MO9, clone 3D8, Abnova Corporation, Taipei, Taiwan) that recognizes a partial recombinant human RasGRP2 (CalDAG-GEFI) protein. The sequence of the recombinant human protein is homologous with canine sequence for CalDAG-GEFI. The antibody was diluted to 1 $\mu$g per mL in TBS-T and reacted with immunoblots for 1 h at 18–24°C. The blots were also probed with a rabbit anti-human Rap1 polyclonal antibody (Stressgen Bioreagents, Victoria, British Columbia, Canada) with cross reactivity to canine Rap1. After incubation with primary antibodies, the blots were washed 3 times in TBS-T, reacted for 1 h at 18–24°C with either purified goat anti-mouse or purified goat anti-rabbit IgG conjugated to HRP (BioRad) diluted 1:50,000 in TBS-T, and then washed 3 times in TBS-T. Immunoreactive protein bands were visualized using the ECL-Plus Western Blotting Detection System (GE Healthcare, Chalfont St. Giles, United Kingdom) and high-performance chemiluminescence film (Hyperfilm ECL; Amersham Biosciences, Piscataway, NJ) according to manufacturer suggested protocols. Films were scanned using standard imaging software (Adobe Photoshop v.7.0.1; Adobe Corporation, San Jose, Calif).

**Platelet phosphoinositides.** Platelets from normal dogs and affected Basset hounds were labeled with [$^3$H]-inositol essentially as detailed by Huang.[20] Radiolabeled platelets were activated by addition of either saline (0.15 M NaCl) or 1 Unit/mL human alpha-thrombin at 37°C, with stirring at 950 RPM for various times from 0 s to 120 s. Reactions were terminated by rapid addition of ice cold 10% (vol/vol) $HClO_4$ and extracted as detailed by Wreggett[21] with inclusion of phytate hydrolysate to optimize recovery. [$^3$H]-labeled inositol phosphates were separated using QMA anion exchange SEP-PAKS (Waters Corp, Milford, Mass) and ammonium formate buffers.[22]

## RESULTS

**Flow cytometry.** Monoclonal antibody Y2/51, recognizing platelet glycoprotein IIIa, bound normally to platelets obtained from affected Basset hounds and Spitz dogs[17] in contrast to platelets obtained from dogs with Glanzmann thrombasthenia (GT) in which binding was markedly reduced.[23] CAP-1, a monoclonal antibody that recognizes a RIBS on canine fibrinogen, did not bind significantly to nonactivated platelets obtained from either normal dogs or dogs with Basset hound thrombopathia or GT[23] (Spitz dog platelets were not available for CAP-1 flow cytometry studies.) Platelets obtained from normal dogs and activated with ADP or PAF bound CAP-1 in a dose-dependent fashion. In contrast, platelets obtained from thrombopathic Basset hounds failed to bind CAP-1 even when activated with ADP concentrations as high as 100 $\mu$M (Fig 2). Platelets from dogs with GT also failed to bind CAP-1.

**CalDAG-GEFI cDNA and DNA sequences.** Platelet-derived cDNA sequences encoding CalDAG-GEFI were evaluated in 2 thrombopathic Basset hounds and 1 normal mixed-breed dog. Sequences obtained from the thrombopathic Basset hounds and the mixed-breed dog matched the Boxer dog sequence available on GenBank except for a 3 base pair deletion (TCT) observed at nucleotides 509, 510, and 511 within Exon 5 (509,510,511*del*TCT) in samples obtained from thrombopathic Basset hounds (Fig 3). Alternatively spliced segments were also observed in cDNA samples from thrombopathic Basset hounds and in normal dogs between Exons 1 and 6. Alternatively spliced versions included portions of Exon 1 and Exon 3 with complete deletion of Exon 2. Exons 4, 5, and 6 within these cDNA sequences were complete except for the missing TCT in Exon 5 noted in samples from thrombopathic Basset hounds. Because of frame shift, premature stop

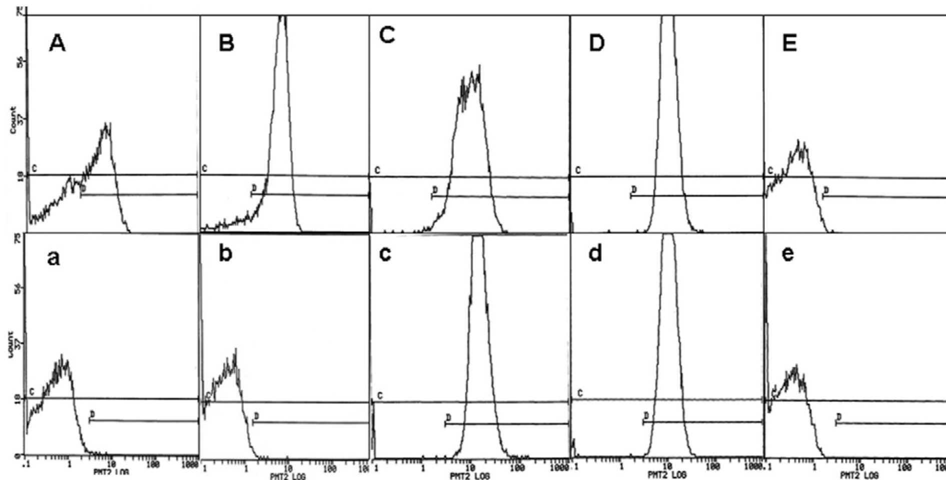


**Fig 2.** Representative flow cytometric results obtained from a normal dog (upper panel, A through E) and a thrombopathic Basset hound (lower panel, a through e). Aa. Mouse anti-RIBS on canine fibrinogen (CAP1) in response to 100 $\mu$M ADP. Bb. Mouse anti-RIBS on canine fibrinogen (CAP1) in response to 0.2 $\mu$M PAF. Cc. Mouse anti-human GPIIb (alpha IIb). Dd. Mouse anti-human GPIIIa (beta 3). Ee. Isotype control. Platelets from Basset hounds with thrombopathia did not bind CAP1, a monoclonal antibody to a RIBS on canine fibrinogen in response to ADP or PAF (**A** and **B**). Similar findings were found using a wide range of ADP and PAF concentrations. Thrombopathic platelets bound antibodies to GPIIb and GPIIIa in a manner similar to normal canine platelets (**C** and **D**). Similar results were found for platelets from a Spitz dog with thrombopathia.

codons appeared within the sequences. The significance of these alternatively spliced cDNA sequences is not known. Unusual 5′ and 3′ splice sites were noted for the intron following Exon 2 (au and ac), which may have contributed to the alternative splicing variations observed. cDNA sequences also revealed a 175 base pair deletion, likely an intron, after the 6th nucleotide following the stop codon, TAA. This deletion was observed in samples from normal dog and from thrombopathic Basset hounds. DNA sequences encoding CalDAG-GEFI were evaluated in 79 Basset hounds, 1 Eskimo Spitz, 8 Landseers, 1 mixed-breed dog, and 1 Cavalier King Charles Spaniel. Of the 79 Basset hounds, 8 were affected (identified either with platelet function studies or by ruling out other possible causes of bleeding). One dog was an obligate carrier and had sired 1 affected dog mentioned above. Platelet function was evaluated in 23 of the remaining Basset hounds, and studies indicated that they either had normal platelet function or slightly reduced platelet function in response to either low doses of ADP, collagen, or both. Platelet function studies were not performed on the remaining Basset hounds who were all reported to be clinically normal. The Eskimo Spitz was an affected dog with a platelet function disorder essentially identical to that described in Basset hounds.[16] Two Landseers were obligate carriers for a bleeding disorder in Landseer in the Netherlands. One Landseers was affected. The remaining 5 Landseers were clinically normal, and 4 were closely related to the affected and obligate carrier Landseers. The mixed-breed dog was clinically normal and had normal platelet function. The Cavalier King Charles Spaniel was clinically normal and had a macro-thrombocytopenia and enhanced platelet reactivity in response to ADP and collagen. DNA sequences for the coding portions of the CalDAG-GEFI gene in 62 Basset hounds were identical to the normal Boxer dog sequence available as part of the dog genome located at NCBI. DNA sequences from the 8 affected Basset hounds were also identical except for a 3 base pair deletion (509,510,511*del*TCT) located in Exon 5 (Fig. 3). This portion of the gene encodes for the structurally conserved region 1 (SCR1) of the catalytic domain within the protein.[24] This deletion would be predicted to result in the elimination of a highly conserved phenylalanine (amino acid 170) from within the catalytic unit of CalDAG-GEFI. The obligate carrier Basset hound was heterozygous for this deletion as were 8 other Basset hounds who were clinically normal but were related to other dogs that had been identified as either being carriers or affected. The other 11, non-Basset dogs were clear of this deletion and matched normal dog genome in this location.

The DNA sequence for the coding region of the CalDAG-GEFI gene in the Eskimo Spitz dog with thrombopathia was identical to the normal Boxer dog sequence except for a single nucleotide insertion (A) between nucleotides 452 and 453 within Exon 5 (452-

```
 Exon 5

    151(452)                                                     170(509-511)
  [F  D  H  L  E  P  L  E  L  A  E  H  L  T  Y  L  E  Y  R  S  F  C  K  I] L
D TTCGACCACCTGGAACCCTTGGAACTAGCAGAGCATCTCACCTACTTAGAGTATCGCTCCTTCTGCAAGATCCTG
B TTCGACCACCTGGAACCCTTGGAACTAGCAGAGCATCTCACCTACTTAGAGTATCGCTCCTXXXGCAAGATCCTG
S TTCGAACCACCTGGAACCCTTGGAACTAGCAGAGCATCTCACCTACTTAGAGTATCGCTCCTTCTGCAAGATCCT
L TTCGACCACCTGGAACCCTTGGAACTAGCAGAGCATCTCACCTACTTAGAGTATCGCTCCTTCTGCAAGATCCTG
M TTCGACCACCTGGAACCCTTGGAACTAGCAGAGCATCTCACCTACTTAGAGTATCGCTCCTTCTGCAAGATCCTG
C TTCGACCACCTGGAACCCTTGGAACTAGCAGAGCATCTCACCTACTTAGAGTATCGCTCCTTCTGCAAGATCCTG

Exon 6                                     Exon 7

  [R  A  L  V  I  T  H  F  V  H  V  A  E  K  L  L  H  L  Q  N  F  N  T  L  M
D CGTGCCCTGGTCATCACGCACTTTGTCCACGTGGCAGAG AAGCTGCTTCACTTGCAGAACTTCAACACTCTGATG
B CGTGCCCTGGTCATCACGCACTTTGTCCACGTGGCAGAG AAGCTGCTTCACTTGCAGAACTTCAACACTCTGATG
S GCGTGCCCTGGTCATCACGCACTTTGTCCACGTGGCAGA GAAGCTGCTTCACTTGCAGAACTTCAACACTCTGAT
L CGTGCCCTGGTCATCACGCACTTTGTCCACGTGGCAGAG AAGCTGCTTCACTTGCAGAACTTCAACACTCTGATG
M CGTGCCCTGGTCATCACGCACTTTGTCCACGTGGCAGAG AAGCTGCTTCACTTGCAGAACTTCAACACTCTGATG
C CGTGCCCTGGTCATCACGCACTTTGTCCACGTGGCAGAG AAGCTGCTTCACTTGCAGAACTTCAACACTCTGATG

                                                                266(796)
   A  V  V  G  G  L  S  H  S  S  I  S  R  L  K  E  T  H] S  H  V  S  S  E  T
D GCCGTGGTCGGAGGCCTGAGCCACAGCTCCATCTCCCGCCTCAAGGAGACTCACAGTCATGTTAGCTCTGAGACC
B GCCGTGGTCGGAGGCCTGAGCCACAGCTCCATCTCCCGCCTCAAGGAGACTCACAGTCATGTTAGCTCTGAGACC
S GGCCGTGGTCGGAGGCCTGAGCCACAGCTCCATCTCCCGCCTCAAGGAGACTCACAGTCATGTTAGCTCTGAGAC
L GCCGTGGTCGGAGGCCTGAGCCACAGCTCCATCTCCCGCCTCAAGGAGACTCACAGTCATGTTAGCTCTGAGACC
M GCCGTGGTCGGAGGCCTGAGCCACAGCTCCATCTCCCGCCTCAAGGAGACTCACAGTCATGTTAGCTCTGAGACC
C GCCGTGGTCGGAGGCCTGAGCCACAGCTCCATCTCCCGCCTCAAGGAGACTCACAGTCATGTTAGCTCTGAGACC

Exon 8

  [G  F  R  F  P  I  L  G  V  H  L  K  D  L  V  A  L  Q  L  A  L  P  D] W  L
D GGTTTCCGCTTTCCTATCCTGGGTGTACACCTCAAGGACCTGGTGGCTCTGCAGCTGGCACTGCCTGACTGGCTG
B GGTTTCCGCTTTCCTATCCTGGGTGTACACCTCAAGGACCTGGTGGCTCTGCAGCTGGCACTGCCTGACTGGCTG
S GGGTTTCCGCTTTCCTATCCTGGGTGTACACCTCAAGGACCTGGTGGCTCTGCAGCTGGCACTGCCTGACTGGCT
L GGTTTCCGCTTTCCTATCCTGGGTGTACACCTCAAGGACCTGGTGGCTCTGCAGCTGGCACTGCCTGACTGGCTG
M GGTTTCCGCTTTCCTATCCTGGGTGTACACCTCAAGGACCTGGTGGCTCTGCAGCTGGCACTGCCTGACTGGCTG
C GGTTTCCGCTTTCCTATCCTGGGTGTACACCTCAAGGACCTGGTGGCTCTGCAGCTGGCACTGCCTGACTGGCTG

                 328(982)
   D  P  A  R  T [R  L  N  G  A  K] M  K  Q  L  F  S  I  L  E  E  L  A  M  V
D GACCCTGCCCGGACCCGACTTAATGGGGCCAAGATGAAGCAGCTCTTCAGCATCCTGGAGGAGCTGGCCATGGTG
B GACCCTGCCCGGACCCGACTTAATGGGGCCAAGATGAAGCAGCTCTTCAGCATCCTGGAGGAGCTGGCCATGGTG
S GGACCCTGCCCGGACCCGACTTAATGGGGCCAAGATGAAGCAGCTCTTCAGCATCCTGGAGGAGCTGGCCATGGT
L GACCCTGCCCGGACCTGACTTAATGGGGCCAAGATGAAGCAGCTCTTCAGCATCCTGGAGGAGCTGGCCATGGTG
M GACCCTGCCCGGACCCGACTTAATGGGGCCAAGATGAAGCAGCTCTTCAGCATCCTGGAGGAGCTGGCCATGGTG
C GACCCTGCCCGGACCCGACTTAATGGGGCCAAGATGAAGCAGCTCTTCAGCATCCTGGAGGAGCTGGCCATGGTG

Exon 9

   D  E [L  Y  Q  L  S  L  Q  R  E  P  R] S  K  S  S
D GATGAACTCTACCAGCTGTCCCTGCAGCGGGAGCCACGCTCCAAGTCCTCG
B GATGAACTCTACCAGCTGTCCCTGCAGCGGGAGCCACGCTCCAAGTCCTCG
S GGATGAACTCTACCAGCTGTCCCTGCAGCGGGAGCCACGCTCCAAGTCCTC
L GATGAACTCTACCAGCTGTCCCTGCAGCGGGAGCCACGCTCCAAGTCCTCG
M GATGAACTCTACCAGCTGTCCCTGCAGCGGGAGCCACGCTCCAAGTCCTCG
C GATGAACTCTACCAGCTGTCCCTGCAGCGGGAGCCACGCTCCAAGTCCTCG
```

**Fig 3.** Partial DNA sequence of the gene encoding CalDAG-GEFI in dogs. Amino acid letter designations are shown for normal canine sequence above each codon. Numbering of amino acids and nucleotides begins with ATG. Nucleotide numbers are in parentheses following the amino acid number for selected codons. D = Boxer, dog genome; B = thrombopathic Basset hound; S = thrombopathic Eskimo Spitz; L = thrombopathic Landseer; M = mixed-breed dog, non-bleeder; C = Cavalier King Charles Spaniel with macrothrombocytopenia and enhanced platelet reactivity. Bracketed section [FDHLEPLELAEHLTYLEYRSFCKI] = SCR1 in Exon 5. Eskimo Spitz dogs with thrombopathia have a single nucleotide insertion (*A*) between nucleic acids 452 and 453 (452-453*ins*A) resulting in a frame shift. Basset hounds with thrombopathia have a 3 nucleotide deletion (XXX = TCT) at nucleotide positions 509,510,511 (509,510,511*del*TCT), which would result in the deletion of a phenylalanine at amino acid position 170. Bracketed section [RALVITHFVHVAEKLLHLQNFNTLMAV-VGGLSHSSISRLKETH] = SCR2 in Exons 6 and 7. Eskimo Spitz dogs with thrombopathia have a premature stop codon appearing at nucleotide position 796 because of a frame shift starting at nucleotide position 453 (S266Stop). Bracketed section [GFRFPILGVHLKDLVALQLALPD] = SCR3 in Exon 8. Bracketed section [RLNGAK] = SCR4 in Exon 8. Landseers with thrombopathia have a single nucleotide substitution at nucleotide position 982 (982C>T) resulting in the appearance of a premature stop codon (R328Stop) within SCR4). Bracketed section [LYQLSLQREPR] = SCR5 in Exon 9.

453*ins*A) at the beginning of the sequence encoding SCR1 of the catalytic domain (Fig 3). This insertion would be predicted to result in a frame shift and encoding of amino acids not compatible with the types of amino acids necessary for the proper function of this protein. Assuming normal splicing, the frame shift would result in the appearance of a premature stop codon near the end of Exon 7. cDNA was not available for evaluation in the affected Spitz. The DNA sequence for the coding region of the CalDAG-GEFI gene in the affected Landseer was identical to the normal Boxer dog sequence except for a nucleotide substitution of T for C at nucleotide position 982 within Exon 8 (982C>T) (Fig 3). This mutation would be predicted to result in the substitution of a premature stop codon in the place of an arginine at amino acid position 328 (R328Stop) within SCR4 of the catalytic unit. This result would greatly impair the function and/or synthesis of the protein. The 2 obligate carrier Landseers were identical to the normal Boxer dog sequence except they were heterozygous for the change described in the affected Landseer. Of the remaining 5 Landseers, 3 were found to be heterozygous for the mutation and 2 were found to match normal dog genome. DNA sequence from a Great Pyrenees dog heterozygous for GT was found to match dog genome at this location. This sequence was evaluated because the Landseer breed was derived from the Great Pyrenees as well as the Newfoundland breed. The DNA sequence for the coding region of the CalDAG-GEFI gene in the Cavalier King Charles Spaniel was identical to the normal Boxer dog sequence except for minor polymorphisms that did not result in a change in encoded amino acids. DNA sequences for the coding region of the CalDAG-GEFI gene in the normal mixed-breed dog matched the normal Boxer dog sequence.

**CalDAG-GEFI expression.** Platelet extracts were evaluated for the presence of CalDAG-GEFI using SDS-PAGE and Western blotting. A murine monoclonal antibody to human CalDAG-GEFI recombinant protein sequence reacted with a protein band at 69 kD that corresponds to the predicted size for CalDAG-GEFI. Platelet extracts from human (n = 1), dog (n = 2), and affected Basset hounds (n = 3) had comparative levels of reactive protein (Fig 4). The blot was also probed for Rap1 using a rabbit polyclonal antibody to human Rap1 with cross reactivity to canine Rap1. Similar amounts of Rap1 were detected in platelet lysates from human, normal dogs, and affected Basset hounds. (Data not shown.) These results demonstrate the expression of CalDAG-GEFI in affected Basset hound platelets and suggest that the mutant protein is dysfunctional. No evidence for enhanced proteolysis of the mutant form of canine CalDAG-GEFI was observed. Platelet lysates were not available from Eskimo Spitz or Landseers; however, in light of their mutations, which result in the appearance of premature stop codons, one could speculate that protein synthesis of platelet CalDAG-GEFI does not occur in those disorders.

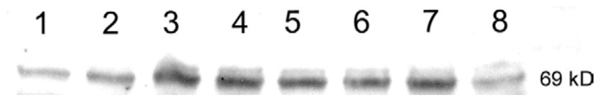


**Fig 4.** Western blot analysis of CalDAG-GEFI in platelet lysates from normal dogs and affected Basset hounds. Each lane was loaded with 40-μg total protein. Lanes 1 and 8 = human, lanes 2 and 6 = normal dog, lanes 3 and 7 = affected Basset hound 1, lane 4 = affected Basset hound 2, and lane 6 = affected Basset hound 3. Canine platelet CalDAG-GEFI was detected using murine monoclonal antibody MO9, clone 3D8 to partial recombinant human Ras-GRP2 (CalDAG-GEFI).

**PAR1, PAR3, and PAR4 cDNA sequences.** Information concerning PAR receptors on canine platelets has not been published, and it is not known whether canine platelets possess PAR1, PAR3, and/or PAR4 receptors similar to those documented in mice and people. This information coupled with the importance of PAR signaling in thrombin mediated platelet activation and the impaired reactivity to thrombin of platelets from dogs with the mutations prompted an evaluation of canine PAR encoding sequences. cDNA sequences encoding PAR1, PAR3, and PAR4 were amplified from cDNA obtained from normal dog platelets and compared with normal human and mouse sequence information. The sequences encoding the cleavage regions of the tethered thrombin receptor activation peptides (TRAPs) suggested that dog platelets do synthesize these 3 receptors similarly to those reported for mouse and human platelets. Encoded amino acids were similar but not identical to either human or mouse platelet receptors (Fig 5). PAR1 cDNA encoded for 2 additional amino acids 15 amino acids beyond the predicted cleavage site that were not present in human sequence. The first 23 amino acids of the predicted tethered ligands for PAR1, PAR3, and PAR4 in dogs were SFFLKNTNDGFEPF-PLEEDEEKN, TFRGAPSNSFEEFPLSAIEGWTE, and SFPGQPWANNSDILEIPESSRAL, respectively.

**Inositol phosphates.** An inherited human bleeding disorder has been identified in association with defective initial platelet reactivity and impaired platelet phosphatidylinositol metabolism.[25] As affected Basset hound platelets exhibited impaired initial platelet reactivity to thrombin, we examined thrombin-stimulated phosphoinositide hydrolysis in normal dogs and affected Basset hounds. Similar levels of radiolabeled $IP_1$, $IP_2$, $IP_3$, and $IP_4$ were extracted after 120 s from

```
PAR1

HUMAN   PR*SFLLRNPNDKYEPFWXXEDEEKN
CANINE  PR*SFFLKNTNDGFEPFPLEEDEEKN

H CCCCGG*TCATTTCTTCTCAGGAACCCCAATGATAAATATGAACCATTTTGGXXXXXXGAGGATGAGGAGAAAAAT
C CCCCGG*TCATTTTTTCTCAAGAATACCAATGATGGATTTGAACCATTCCCACTGGAGGATGATGAGGAGAAAAAT

*Cleavage site for human and dog (activation peptides are SFLLRN or SFFLKN,
respectively)

PAR4

HUMAN   PR*GYPGQVCANDSDTLELPESSRAL
CANINE  LR*SFPGQPWANNSDILEIPESSRAL
MURINE  PR*GYPGKFCANDSDTLELPASSQAL

H CCCCGC*GGCTACCCAGGCCAAGTCTGTGCCAATGACAGTGACACCCTGGAGCTCCCGGACAGCTCACGGGCACTG
C CTGCGC*AGCTTCCCCGGCCAGCCCTGGGCTAACAACAGCGAGATCTTGGAGATCCCAGAAAGCTCCCGCGCCCTG
M CCACGA*GGCTACCCGGGCAAATTCTGTGCCAACGACAGTGACACGCTGGAGCTCCCGGCCAGCTCTCAAGCACTG

*Cleavage sites for human, dog, and mouse (activation peptides are GYPGQV,
SFPGQP, or GYPGKF, respectively)

PAR3

HUMAN   IK*TFRGAPPNSFEEFPFSALEGWTX
CANINE  IK*TFRGAPSNSFEEFPLSAIEGWTE
MURINE  IK*SFNGAPQNTFEEFPLSDIEGWTX

H ATTAAG*ACCTTTCGTGGAGCTCCCCCAAATTCTTTTGAAGAGTTCCCCTTTTCTGCCTTGGAAGGCTGGACAXXX
C ATCAAG*ACCTTCCGTGGGGCTCCCTCAAATTCTTTTGAAGAGTTCCCCCTTTCTGCCATAGAAGGCTGGACAGAA
M ATTAAG*AGTTTTAATGGGGGTCCCCAAAATACCTTTGAAGAATTCCCACTTTCTGACATAGAGGGCTGGACAXXX
*Cleavage sites for human, dog, and mouse (activation peptides are TFRGAP,
TFRGAP, or SFNGAP, respectively)
```

**Fig 5.** Partial cDNA sequences encoding PAR1, PAR4, and PAR3 in dog (C) compared to human (H) and mouse (M) sequences obtained from GenBank. Encoded amino acids (letter designations) are shown. cDNA sequences encompass regions encoding the cleavage sites for the activation peptides. Cleavage sites determined for mouse and human and proposed cleavage sites for dog are indicated (*). Activation peptides are underlined. X's are used to designate absence of nucleotides or amino acids observed during sequence alignment.

unstimulated saline control platelets evaluated in normal and affected dogs. At 120 s, levels of $IP_1$, $IP_2$, $IP_3$, and $IP_4$ were 1342, 1300; 497, 390; 226, 260 and 226, 236 [$^3$H] dpm/$10^9$ platelets for normal and affected dogs, respectively. In contrast, at all time points, postthrombin activation (5, 10, 15, 30, and 120 s) IP extracts from affected platelets were 1.5-fold to 2-fold higher, with peak thrombin-stimulated accumulation at 120 s. $IP_1$, $IP_2$, $IP_3$, and $IP_4$ levels at 120 s postthrombin stimulation were 2787, 5390; 2558, 2830; 557, 873 and 180, 378 [$^3$H] dpm/$10^9$ platelets from normal and affected dogs, respectively.

## DISCUSSION

Inherited platelet function disorders in Basset hounds and Spitz dogs have been reported previously.[15-17] Platelet aggregation responses of affected Basset hounds and Spitz dogs are essentially identical and are characterized by normal shape change responses but markedly impaired aggregation responses to ADP, collagen, calcium ionophore A23187, and PAF. In both disorders, the thrombin response is delayed with maximal aggregation occurring in approximately 5 min to 6 min instead of the typical 2-min to 3-min maximum response observed with normal canine platelets (Fig 6).

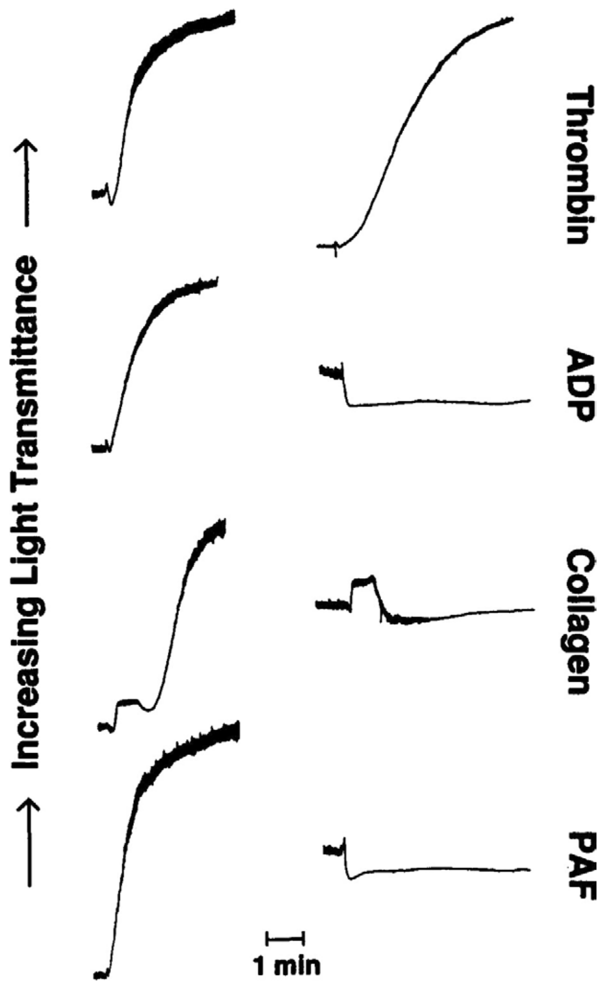


**Fig 6.** Representative aggregation tracings from Basset hounds or Spitz dogs with thrombopathia compared with normal dogs. Aggregation responses in affected dogs are markedly impaired at all concentrations of agonists used. Thrombopathic platelets respond to thrombin with a characteristic lag phase. Reprinted with permission from Boudreaux et al.[17] Copyright 1994 Journal of Veterinary Internal Medicine.

Clot retraction is normal in both disorders. Dense granule secretion as demonstrated using $^{14}$C-serotonin release is markedly impaired in thrombopathic platelets obtained from Basset hounds and Spitz dogs.[16,17] Although uptake of serotonin is normal, thrombopathic platelets release less than 6% of their total platelet $^{14}$C-serotonin in response to collagen or A23187. ADP-induced secretion is highly variable between dogs; however, the kinetics of release is unique and very unusual in thrombopathic platelets obtained from Basset hounds. Thrombopathic platelets release their $^{14}$C-serotonin within 30 s of ADP addition, and the percent released is dose-independent. In contrast, normal dog platelets do not release $^{14}$C-serotonin before 1-min post-ADP addition and percent release is dose-dependent. Thrombin-induced $^{14}$C-serotonin release is comparable in thrombopathic and normal dog platelets. In earlier studies, thrombopathic Basset hound platelets were found to have increased basal levels of cAMP as well as impaired cAMP metabolism in experiments using an activator of adenylate cyclase (forskolin) and a phosphodiesterase (PDE) inhibitor, 1-methyl-3-isobutylxanthine.[26,27] PDE activity was normal in platelet extracts, which suggested an impairment of regulatory control. Platelets from affected Basset hounds also exhibited increased levels of extractable phosphoinositides when stimulated with thrombin, which suggested either differences in phosphoinositide metabolic pathways or differences in PI anchoring and recovery. These findings suggest a putative link between CalDAG-GEFI and its target Rap1 or an unrecognized target Ras involved in pathways that regulate cAMP PDE activity and thrombin-stimulated phosphoinositide anchoring or metabolism. In this study, 3 distinct mutations were identified in 3 different breeds of dogs in the gene encoding CalDAG-GEFI, a guanine nucleotide exchange factor found in platelets that is critically important for normal platelet function. These mutations were all located within regions of the gene encoding structurally conserved regions within the catalytic domain of the protein. In affected Basset hounds, Western blots of platelet lysates indicated that CalDAG-GEFI levels were similar to normal dogs, which suggests that this mutation results in expression of a protein that seems to be functionally impaired. The 3 distinct mutations identified in affected dogs are associated with profound platelet dysfunction and abnormal bleeding tendencies that parallel those recently reported for CalDAG-GEFI knockout mice (Table I). The significance of CalDAG-GEFI function was demonstrated in studies with megakaryocytes that did not have the transcription factor NF-E2. NF-E2 $^{-}/^{-}$ megakaryocytes could not undergo agonist-induced fibrinogen binding[28] because of the absence of the gene encoding CalDAG-GEFI.[29] Forced expression of CalDAG-GEFI in these megakaryocytes led to enhanced agonist-induced binding of fibrinogen to alphaIIb-beta3.[30] CalDAG-GEFI contains 4 major domain structures including (1) Ras exchanger motif domain common to Ras family GEFs; (2) cdc25-like GEF (catalytic) domain; (3) 2 EF hand domains for interaction with calcium; and (4) C1 domain for interaction with DAG and phorbol esters.[30] The catalytic domain of CalDAG-GEFI is critically important for the GEF-mediated activation of Rap1b.

Thrombopathic platelets undergo normal clot retraction and respond fully to thrombin although with impaired kinetics. PARs have not been characterized on canine platelets. In a comparative study, dog platelets were found to be nonresponsive to SFLLRN.[31,32] This nonresponsiveness may have been from use of the nonspecific peptide for dog platelets (SFLLRN instead

**Table I.** Comparative phenotype data for CalDAG-GEFI knockout mice and dogs homozygous for CalDAG-GEFI gene mutations

| TESTS | CalDAG-GEFI knockout mice | CalDAG-GEFI gene mutations (dogs) |
|---|---|---|
| Platelet number | normal | normal |
| Bleeding time | markedly prolonged | markedly prolonged |
| Coagulation assays | normal | normal |
| Intraplatelet/plasma fibrinogen | not evaluated | normal |
| von Willebrand factor | not evaluated | normal |
| Tether to a collagen surface | normal | normal |
| Firmly anchor to a collagen surface | impaired | impaired* |
| Platelet function: | | |
| Shape change (all tested agonists) | normal | normal |
| Aggregation: | | |
| ADP, 2–10 uM | absent | absent |
| ADP, > 50 uM | absent | absent; occasional microaggregates |
| Collagen, > 20 ug/uL | absent | absent |
| alpha thrombin, 0.1 U/mL | rate and max extent impaired | rate and max extent impaired |
| alpha thrombin, 1.0 U/mL | rate and max extent normal | rate impaired, max extent normal |
| gamma thrombin, 16 nM | not evaluated | rate impaired, max extent normal |
| Calcium Ionophore A23187 | | |
| 5–10 uM in PRP | absent | absent |
| 5–10 uM in GFP + 1 mM Ca2+ | not evaluated | rate impaired, max extent normal |
| U46619 or Na Arachidonate | impaired | impaired |
| PMA, 1.5–3 uM | normal | normal |
| Adenine nucleotide content | not evaluated | normal |
| Granule secretion | | |
| ADP, 10 to 100 uM | not evaluated | atypical |
| Collagen, 12–100 ug/mL or CRP 1–50 ug/mL | impaired | impaired |
| alpha thrombin, 0.1 U/mL | normal | normal |
| alpha thrombin, 1.0 U/mL | normal | normal |
| αIIbβ3 integrin concentration | normal | normal |
| αIIbβ3 integrin activation | | |
| ADP, 10 to 100 uM | not evaluated | impaired |
| Collagen, 12 to 100 ug/mL or CRP 1–50 ug/mL | impaired | impaired |
| alpha thrombin, 0.1 U/mL | normal | impaired |
| alpha thrombin, 1.0 U/mL | not evaluated | normal |
| Calcium Ionophore A23187, 1 uM | not evaluated | impaired |
| Calcium Ionophore A23187, 10 uM | not evaluated | normal |
| Clot retraction | not evaluated | normal |
| Intraplatelet cAMP | | |
| Resting, unstimulated | not evaluated | slightly elevated |
| Forskolin stimulated | not evaluated | markedly elevated |
| cAMP PDE activity, regulatory control | not evaluated | impaired |
| Extractable phosphoinositides | | |
| alpha thrombin, 1.0 U | not evaluated | elevated |

*Prolonged collagen-induced thrombus formation (Clot signature analyzer, unpublished data).

of SFFLKN) or from reduced affinity of binding of soluble TRAPs to canine PAR receptors on platelets. The possibility of the latter is suggested by parallel dose response curves obtained for platelet activation of canine or human platelets by human alpha thrombin[16] and human gamma thrombin (Catalfamo, unpublished data), which has been reported to selectively activate PAR4.[33] Our evaluation of dog platelet cDNA sequences suggests that dog platelets do possess PAR1, PAR3, and PAR4 receptors. Biphasic kinetics of activation and signaling for PAR1 and PAR4 have been described for human platelets.[34] In that study, PAR1 binding resulted in a rapid spike in calcium influx followed by a PAR4-induced slower and prolonged calcium influx. PAR4 was found to generate a sustained calcium signal and was considered to be more effective than PAR1 in eliciting secondary autocrine signals necessary for complete platelet activation. The delayed kinetics of thrombin activation observed in thrombopathic canine platelets may be mediated by PAR4 sig-

naling. This result suggests that PAR1 signaling may require CalDAG-GEFI activation of Rap1b, whereas PAR4 signaling does not. Thrombin has been shown to activate Rap1b in 2 phases, with the second phase being mediated via protein kinase C (PKC).[35] Phorbol myristate acetate, an activator of PKC, has been shown to activate platelets of thrombopathic Basset hounds in a manner similar to normal canine platelets.[36] These findings suggest that thrombin signaling through PAR4 is linked to activation of PKC with resulting activation of Rap1b in a manner independent of CalDAG-GEFI. Thrombin-induced serotonin release kinetics were rapid and normal in thrombopathic platelets, which rules out delayed dense granule release of ADP as a reason for the delay in thrombin-induced platelet aggregation. Thrombopathic platelets did not bind CAP-1, a monoclonal antibody to a RIBS epitope on canine fibrinogen, in response to ADP or PAF, even at high agonist concentrations. In an earlier study,[37] thrombopathic platelets were found to bind soluble fibrinogen in the absence of platelet aggregation. In that study it is likely that the form of fibrinogen detected on platelet surfaces had not undergone the conformational change induced by binding to the alphaIIb-beta3 integrin receptor that is detected by CAP-1. Interestingly, thrombopathic platelets do form micro-aggregates in the presence of high concentrations of ADP. ADP may be able to induce slight affinity changes in the integrin receptor that allows some fibrinogen binding; however, in the absence of CalDAG-GEFI effects, the receptor cannot complete the conformational change that results in the change in the conformation of bound fibrinogen. This interpretation is consistent with the failure of activated thrombopathic platelets to interact with bead immobilized fibrinogen.[16] Changes in ligand conformation likely play a key role in outside-in signaling events through the alphaIIb-beta3 integrin that lead to complete affinity and avidity modulation necessary for full platelet aggregation and eventual clot retraction. The relationship between dysfunctional CalDAG-GEFI and impaired cAMP metabolism identified in platelets obtained from thrombopathic Basset hounds is interesting. It is possible that platelet Rap1b or other CalDAG-GEFI-activated signaling molecules have roles not previously recognized in pathways that regulate PDE activity and cAMP levels. It is also possible that CalDAG-GEFI serves a scaffolding function or acts as a binding partner for PDE and other signal transduction molecules in addition to its function as a GEF for Rap1b. This type of relationship has been documented in other cell types, including cardiac myocytes in which muscle A kinase-anchoring protein acts as a scaffolding protein for PDE4D3, protein kinase A, and Epac1, a guanine nucleotide exchange factor activated by cAMP.[38] It would be informative to evaluate cAMP metabolism in CalDAG-GEFI knockout mice.

Three distinct mutations were identified in the gene encoding CalDAG-GEFI. These mutations were all located within regions of the gene encoding structurally conserved regions within the catalytic domain of the protein. As a result of these mutations, affected dogs experienced a profound degree of platelet dysfunction and abnormal bleeding tendencies. The similarity of the platelet dysfunction observed in affected dogs to those of knockout mouse was striking. The finding of mutations in 3 different dog breeds with platelet dysfunction suggests that functional mutations in the CalDAG-GEFI gene may be associated with similar defects in human patients. This finding is underscored by the realization that most human patients with congenital platelet disorders have primary secretion defects of unknown etiology.[39] CalDAG-GEFI has been demonstrated to play a critical role in mouse and canine function. This protein is involved in signal transduction events that are important for inside-out as well as outside-in integrin signaling events. The protein also may play an important role in regulating the platelet release reaction and likely plays a role in cAMP metabolism and PDE regulation. CalDAG-GEFI may be a new target for drugs designed and developed to regulate platelet function. Future studies will be aimed at evaluating CalDAG-GEFI function in thrombopathic Basset hound platelets. Efforts will also be made to evaluate CalDAG-GEFI expression in thrombopathic Landseer platelets if materials become available.

The authors wish to thank Kevin King for technical assistance with this project and the Basset hound breeders who submitted samples for this study, including Celeste Gonzalez, Carolyn Young, Joan Urban, Claudia Orlandi, and Barbara Kahl.

## REFERENCES

1. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002;110:673–87.
2. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. Blood 2004;104:1606–15.
3. Bos JL. All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. EMBO J 1998;17:6776–82.
4. Lapetina EG, Lacal JC, Reep BR, Molina y Vedia L. A ras-related protein is phosphorylated and translocated by agonists that increase cAMP levels in human platelets. Proc Natl Acad Sci U S A 1989;86:3131–4.
5. Fischer TH, Gatling MN, Lacal JC, White GC. Rap1B, a cAMP-dependent protein kinase substrate, associates with the platelet cytoskeleton. J Biol Chem 1990;265:19405–8.
6. Franke B, Akkerman JWN, Bos JL. Rapid calcium mediated activation of Rap1 in human platelets. EMBO J 1997;16:252–9.
7. Torti M, Lapetina EG. Role of rap1B and p21ras GTPase-activating protein in the regulation of phospholipase C-gamma 1

in human platelets. Proc Natl Acad Sci U S A 1992;89: 7796–800.
8. Lova P, Paganini S, Sinigaglia F, Balduini C, Torti M. A Gi-dependent pathway is required for activation of the small GTPase Rap1B in human platelets. J Biol Chem 2002;277:12009–15.
9. Woulfe D, Jiang H, Mortensen R, Yang Y, Brass LF. Activation of Rap1B by Gi family members in platelets. J Biol Chem 2002;277:23382–90.
10. Bernardi B, Guidetti GF, Campus F, Crittenden JR, Graybiel AM, Balduini C, et al. The small GTPase Rap1b regulates the cross talk between platelet integrin $\alpha_2\beta_1$and integrin $\alpha_{IIb}\beta_3$. Blood 2006;107:2728–35.
11. Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White II GC: Rap1b is required for normal platelet function and hemostasis in mice. J Clin Invest 2005;115:680–7.
12. Boriack-Sjodin PA, Margarit SM, Bar-Sagi D, Kuriyan J. The structural basis of the activation of Ras by Sos. Nature 1998; 394:337–43.
13. Crittenden JR, Bergmeier W, Zhang Y, Piffath CL, Liang Y, Wagner DD, et al. CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nat Med 2004;10:982–6.
14. Bertoni A, Tadokoro S, Eto K, Pampori N, Parise LV, White GC, et al. Relationship between Rap1b, affinity modulation of integrin $\alpha_{IIb}\beta_3$, and the actin cytoskeleton. J Biol Chem 2002;277: 25715–21.
15. Johnstone IB, Lotz F. An inherited platelet function defect in basset hounds. Can Vet J 1979;20:211–5.
16. Catalfamo JL, Raymond SL, White JG, Dodds WJ. Defective platelet-fibrinogen interaction in hereditary canine thrombopathia. Blood 1986;67:1568–77.
17. Boudreaux MK, Crager C, Dillon AR, Stanz K, Toivio-Kinnucan M. Identification of an intrinsic platelet function defect in Spitz dogs. J Vet Int Med 1994;8:93–8.
18. Boudreaux MK, Panangala VS, Bourne C. A platelet activation-specific monoclonal antibody that recognizes a receptor-induced binding site on canine fibrinogen. Vet Pathol 1996;33:419–27.
19. Brooks MB, Catalfamo JL, Brown HA, Pavlina I, Lovaglio J. A hereditary bleeding disorder of dogs caused by lack of platelet procoagulant activity. Blood 2002;99:2434–41.
20. Huang EM, Detwiler TC. Thrombin-induced phosphoinositide hydrolysis in platelets. Receptor occupancy and desensitization. Biochem J 1987;242:11–8.
21. Wreggett KA, Howe LR, Moore JP, Irvine RF. Extraction and recovery of inositol phosphates from tissues. Biochem J 1987; 245:933–4.
22. Wreggett KA, Irvine RF. A rapid separation method for inositol phosphates and their isomers. Biochem J 1987;245:655–60.
23. Boudreaux MK, Kvam K, Dillon AR, Bourne C, Scott M, Schwartz KA, et al. Type I Glanzmann's thrombasthenia in a Great Pyrenees dog. Vet Pathol 1996;33:503–11.
24. Quilliam LA, Rebhun JF, Castro AF. A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. Prog Nuc Acid Res Mol Biol 2002;71: 391–444.
25. Lages B, Weiss HJ. Impairment of phosphatidylinositol metabolism in a patient with a bleeding disorder associated with defects in initial platelet responses. Thromb Haemost 1988;59: 175–9.
26. Boudreaux MK, Dodds WJ, Slauson DO, Catalfamo JL. Evidence for regulatory control of canine platelet phosphodiesterase. Biochem Biophys Res Commun 1986;140:589–94.
27. Boudreaux MK, Dodds WJ, Slauson DO, Catalfamo JL. Impaired cAMP metabolism associated with abnormal function of thrombopathic canine platelets. Biochem Biophys Res Commun 1986;140:595–601.
28. Shiraga M, Ritchie A, Aidoudi A, Baron V, Wilcox D, White G, et al. Primary megakaryocytes reveal a role for transcription factor NF-E2 in integrin alpha IIb beta 3 signaling. J Cell Biol. 1999;147:1419–1430.
29. Kawasaki H, Springett GM, Toki S, Canales JJ, Harlan P, Blumenstiel JP, et al. A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. Proc Natl Acad Sci U S A 1998;95:13278–83.
30. Eto K, Murphy R, Kerrigan SW, Bertoni A, Stuhlmann H, Nakano T, et al. Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling. Proc Natl Acad Sci U S A 2002;99:12819–24.
31. Catalfamo JL, Andersen TT, Fenton JW II. Thrombin receptor-activating peptides unlike thrombin are insufficient for platelet activation in most species [abstract]. Thromb Haemost 1993;69: 1195.
32. Derian CK, Santulli RJ, Tomko KA, Haertlein BJ, Andrade-Gordon P. Species differences in platelet responses to thrombin and SFLLRN. Receptor-mediated calcium mobilization and aggregation, and regulation by protein kinases. Thromb Res 1995; 78:505–19.
33. Soslau G, Goldenburg SJ, Class R, Jameson B. Differential activation and inhibition of human platelet thrombin receptors by structurally distinct alpha, beta- and gamma-thrombin. Platelets 2004;15:155–66.
34. Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. Biochem 2000;39:5458–67.
35. Franke B, van Triest M, De Bruijn KMT, van Willigen G, Nieuwenhuis HK, Negrier C, et al. Sequential regulation of the small GTPase Rap1 in human platelets. Mol Cell Biol 2000;20: 779–85.
36. McConnell MF, Thomas JS, DiPinto MN, Bell TG. Circumvention of the Basset hound hereditary thrombopathy by platelet activation with phorbol myristate acetate. Platelets 1995;6:131–45.
37. Patterson WR, Estry DW, Schwartz KA, Borchert RD, Bell TG. Absent platelet aggregation with normal fibrinogen binding in Basset hound hereditary thrombopathy. Thromb Haemost 1989; 62:1011–15.
38. Dodge-Kafka KL, Kapiloff MS. The mAKAP signaling complex: Integration of cAMP, calcium, and MAP kinase signaling pathways. European J Cell Biol 2006;85:593–602.
39. Rao AK, Gabbeta J. Congenital disorders of platelet signal transduction. Arterioscler Thromb Vasc Biol 2000;20:285–9.