Rab geranylgeranyl transferase α mutation in the gunmetal mouse reduces Rab prenylation and platelet synthesis


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Few molecular events important to platelet biogenesis have been identified. Mice homozygous for the spontaneous, recessive mutation gunmetal (gm) have prolonged bleeding, thrombocytope- nia, and reduced platelet α- and δ-granule contents. Here we show by positional cloning that gm results from a G → A substitution mutation in a splice acceptor site within the α-subunit of Rab geranylgeranyl transferase (Rabggtα), an enzyme that attaches geranylgeranyl groups to Rab proteins. Most Rabggtα mRNAs from gm tissues skipped exon 1 and lacked a start codon. Rabggtα protein and Rab geranylgeranyl transferase (GGTase) activity were reduced 4-fold in gm platelets. Geranylgeranyltransferase and membrane association of Rab27, a Rab GGTase substrate, were significantly decreased in gm platelets. These findings indicate that geranylgeranylation of Rab GTPases is critical for hemostasis. Rab GGTase inhibition may represent a new treatment for thrombocytope- nia, and clotting disorders.

Platelets play a pivotal role in acute myocardial infarction, unstable angina, deep venous thrombosis, and stroke. About 4 million individuals are hospitalized each year in the United States with these disorders. Treatment with agents that prevent platelet activation reduces the risk of myocardial infarction and stroke by about 30% and death by about 15% (1). Novel platelet antagonists are needed, however, because platelet activation is not always inhibited by current drugs.

Mice homozygous for gunmetal (gm), a spontaneous, recessive mutation, have prolonged bleeding caused by defects in platelets and megakaryocytes (2, 3). These mice also have macrothrombocytopenia, and reduced platelet α- and δ-granule contents (storage pool deficiency, SPD). Megakaryocytes, the progenitors of platelets, are more plentiful in gm mice, but have abnormal intracellular membranes, increased emperipolesis, and decreased platelet synthesis. In addition, gm homozygotes have partial cutaneous albinism (Fig. 1). These features closely resemble the rare human disorders gray platelet syndrome (GPS) and platelet α,δ-SPD. Positional cloning of gm was undertaken both to shed light on the mechanism of disease in platelet SPD disorders and to identify a possible target for antiplatelet drug development.

Materials and Methods

Mice and Genetic Mapping. Mice obtained from The Jackson Laboratory were bred at Roswell Park Cancer Institute. Backcross mice were phenotyped by coat color and genotyped for simple sequence length polymorphisms (SSLPs) and restriction fragment length polymorphism (RFLP) using standard techniques. Linkage relationships were determined by segregation analysis, and best locus order was decided by minimizing crossovers and eliminating double crossovers (4).

Physical Mapping. Mouse yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) clones were identified from libraries (Research Genetics, Huntsville, AL) by PCR or hybridization with oligonucleotides corresponding to chromosome 14 loci. Clones were oriented by sequence-tagged site content mapping and insert end-sequencing. Additional markers were isolated by inverse repetitive element PCR (5).

Inverse Repetitive Element-Direct cDNA Selection. Expressed sequences were isolated from YACs by inverse repetitive element-direct cDNA selection (6). Briefly, outward-oriented, biotin-labeled, B1 repetitive element-specific primers were used for long-range PCR of YAC clones (5). Denatured PCR products were incubated with ribosomal DNA, Cot-1 DNA, and yeast DNA, and then with denatured mouse bone marrow cDNA (attached to amplification cassettes). PCR products (and annealed cDNAs) were captured on streptavidin-coated beads and washed stringently, and cDNAs were eluted. Eluted cDNAs were reamplified and used in a second selection cycle. Resultant cDNA fragments were subcloned and sequenced.

Full-Length Rabggtα (α-Subunit of Rab Geranylgeranyl Transferase) cDNA Isolation. 5′ and 3′ Rapid amplification of cDNA ends (RACE) PCR was performed with mouse fetal brain cDNA (CLONTECH). 5′ RACE used a Rabggtα-specific reverse primer (5′-TTCAACGCGAGACGTCTCTC-3′) and universal forward primer, followed by a second, nested PCR with another Rabggtα primer (5′-CTCTAGCTGTTTGGCCTCGT-3′) and second universal primer. 3′ RACE was performed by using a Rabggtα-specific forward primer (5′-CCAGCAGTCTGCTGCACTTC-3′) and universal reverse primer, and a second, nested PCR with the Rabggtα primer (5′-GGAGGCGTTTTGGGTAGATG-3′) and another universal reverse primer. Products were subcloned and sequenced, and full-length Rabggtα cDNA sequence was assembled.

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Abbreviations: gm, gunmetal; Rabggtα, α-subunit of Rab geranylgeranyl transferase; GGTase, geranylgeranyl transferase; CHM, choroideremia; GPS, gray platelet syndrome; SPD, storage pool deficiency; YAC, yeast artificial chromosome; BAC, bacterial artificial chromosome; RT-PCR, reverse transcription–PCR; REP, Rab escort protein; [3H]GGPP, [3H]geranylgeranyl pyrophosphate.

Data deposition: The sequences reported in this paper have been deposited in the Mouse Genome Database (accession nos. J:52722 and J:46919) and the GenBank database (accession nos. AF127654, AF127655, AF127656, AF127660, and AF127661).

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Reverse Transcription–PCR (RT-PCR) Analysis. Total RNA was prepared from mouse tissues with equilibrated phenol/guanidine isothiocyanate (GIBCO/BRL). Oligo(dT)-primed, first-strand cDNA was synthesized with SuperScript II (GIBCO/BRL). All Rabggta exons were amplified by PCR with gene-specific primers. Aberrant gm splicing was identified with Rabggta primers 5′-GTGCAAGGGTCCACGGGAC-3′ and 5′-TTCAACACG-CAGACAGCTCT-3′.

Western Blot Analysis. Platelet and bone marrow protein were isolated as described (7) except that platelets were washed twice with 0.38% sodium citrate in 0.85% NaCl and once with 1% ammonium oxalate to remove residual red blood cells. Twenty micrograms of protein in proteinase inhibitor mixture was incubated in SDS/mercaptoethanol and electrophoresed on denaturing 10% polyacrylamide gels, followed by Western blotting on nitrocellulose or poly(vinylidene difluoride) membranes (2). For subcellular fractionation studies, fresh platelets were sonicated and separated into membrane and soluble subcellular fractions by centrifugation as described (2). Samples were immediately separated on 12% polyacrylamide gels. Western blots were probed with specific antisera against Rabggta (8) or Rab27 (9), followed by peroxidase-labeled goat α-rabbit IgG secondary antibody (Kirkegaard & Perry Laboratories), and visualized by incubation with ECL-Plus reagent (Amersham Pharmacia) and autoradiography. Several exposures were taken and comparisons were made in the linear range. Blots were washed and reprobed with antibodies to mouse actin. Alternatively GTP-binding proteins were detected on Western blots by hybridization with [32P]GTP, washing, and autoradiography (2).

Assay of GGase I Activity. GGase I activity was determined by measuring 3H transfer from [3H]GGPP to Rac1 in a 25-μl reaction containing 50 mM sodium Hepes (pH 7.2), 150 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1.2 μM [3H]GGPP, 200 μM Zwittergent 3–14, 25 μM recombinant Rac1, and the indicated amounts of cytosolic proteins (9). After incubation for 30 min at 37°C, ethanol/HCl precipitable radioactivity was measured.

Results and Discussion

Genetic and Physical Mapping of gm. Previously, gm had been localized to a 1.5-centimorgan (cM) interval on mouse chromosome 14 (10). Genotyping of 29 loci in 2,462 gm backcross mice narrowed the gm critical region to 0.64 cM between D14Mit63 and D14Mit122 (Fig. 2). Three backcrosses were used to minimize the effect of a local recombination cold spot observed in a Mus spretus backcross. Genetic markers flanking and within the gm critical region were used to isolate contiguous YAC and BAC clones encompassing gm. To complete the cloning of the interval, novel, polymorphic simple sequence repeats (D14SfK1, D14SfK3, and D14SfK5) and sequence-tagged sites were isolated from critical region YACs by inverse repetitive element-PCR (5). They were mapped and used to isolate additional YACs and BACs to cover the interval (Fig. 2). A family of serine protease genes (Mcpt1, Mcpt2, Mcpt4, Mcpt5, Mcpt9, McptL, Cita1, Cita5, Cita6, Cita7, and Ctsg) were mapped to the gm critical region but excluded from gm candidacy by virtue of incompatible distribution of expression or absence of mutations (data not shown).

Candidate Gene Isolation. Additional candidate genes were identified from pooled gm contig YACs and BACs by using a modified direct cDNA selection method (6). Nineteen of 160 selected cDNA fragments were derived from Rabggta. Rabggta-derived sequence-tagged sites were physically mapped on six gm critical region YAC and BAC clones (Fig. 2). A 2.2-kb M. spretus BamHI restriction fragment length polymorphism enabled genetic mapping of Rabggta in the M. spretus backcross. Rabggta cosegregated with gm (Fig. 2), confirming derivation from the gm critical region.

Because mouse Rabggta was novel, cDNAs corresponding to the full-length coding domain were isolated and sequenced. The mouse cDNA shared 94% and 86% nucleotide identity to rat and human Rabggta, respectively.

Rabggta Mutation Identification. Analysis of the genomic sequence of Rabggta revealed a single difference between C57BL/6J-+/+ and C57BL/6J-gm/gm DNA: The normal 3′ terminal nucleotide of intron α (guanine) was replaced with an adenine in gm DNA (Fig. 3). This substitution created a Tsp509I restriction site that was used to show perfect cosegregation of Rabggta and gm in 2,462 meioses.

Rabggta RNA Splicing Defects. Typically, substitution of the obligatory 3′ terminal guanine of the consensus splice acceptor site prevents normal mRNA splicing and leads to intron retention, exon skipping, or cryptic splice site utilization (11). RT-PCR of the 5′ end of Rabggta RNA (corresponding to exons α, 1, and 2) disclosed aberrantly spliced Rabggta transcripts in gm bone marrow (Fig. 3). This tissue was used because the hematologic abnormalities of gm homozygotes are corrected by bone marrow transplantation (3). In normal mice, RT-PCR of the 5′ end of Rabggta gave two products. The most abundant product was correctly spliced (exon α - exon 1 - exon 2; Fig. 3, +/+ product a). A second, minor product in normal mice retained intron α (exon α - intron α - exon 1 - exon 2; Fig. 3, +/+ product b) and
may be immature, because it could only be marginally detected by RT-PCR with cytosolic or poly(A)+ RNA (data not shown).

In gm homozygotes, the major Rabggtb RT-PCR product of +/+ bone marrow was not observed (product a, exon α - exon 1 - exon 2). Instead, the most abundant gm product skipped Rabggtb exon 1 (Fig. 3, gm/gm product c; exon α - exon 2). Translation of this transcript was not anticipated to yield functional Rabggtb protein because the initiation codon was absent, and no alternative, in-frame start codon was present (Fig. 3). A second novel, but much less abundant, gm Rabggtb RT-PCR product represented activation of a cryptic splice donor (within intron α, 84 bp downstream of the normal splice donor site) and acceptor (within exon 1, 13 bp downstream of the G→A substitution) sites (Fig. 3, gm/gm product d). Utilization of these cryptic splice sites did not result in removal of the initiation codon, and the existence of this transcript may explain the presence of residual Rabggtb in gm homozygotes.

Like +/+ bone marrow, a rare product that retained intron α occurred in gm/gm RNA (exon α - intron α - exon 1 - exon 2; Fig. 3, product b′). This gm/gm transcript also was anticipated to be functional if polyadenylated, because the initiation codon of Rabggtb is in exon 1, and therefore retention of gm intron α (with G→A substitution) should not disrupt translation. However, RT-PCR of cytosolic or poly(A)+ RNA suggested that it was an immature, nonpolyadenylated RNA, like the corresponding transcript in +/+ bone marrow (data not shown).

These aberrant Rabggtb splice variants were found in all tissues tested (gm bone marrow, melanocyte, and kidney; data not shown). As expected, both normal and mutant products were observed in gm/+ heterozygote RNA (data not shown). No other defect in splicing was observed in gm/gm; other regions of Rabggtb mRNA produced RT-PCR products of expected size and sequence, with the exception of a Rabggtb isoform that retained intron 9 (in both +/+ and gm/gm mice).

The enzyme Rab GGTase is composed of Rabggtb and a β-subunit (Rabggtb) and absolutely requires Rab escort protein (Rep) 1 or 2 for activity (12, 13). The gm reduction in Rabggtb was specific. Quantitative RT-PCR revealed no changes in the abundance of transcripts of Rabggtb, Rep1 or Rep2 in gm/gm bone marrow or kidney (data not shown). Furthermore, Northern blots disclosed no difference between gm/gm and +/+ tissues in the overall abundance of Rabggtb mRNA (data not shown).

**Rabggtb Protein Abundance.** Western blots of gm platelets showed one consequence of aberrant Rabggtb mRNA splicing to be ~70% reduction in 60-kDa Rabggtb protein in gm platelets compared with +/+ (Fig. 4). It is likely that residual Rabggtb protein in gm/gm platelets resulted from translation of the novel gm Rabggtb transcript that used cryptic splice sites (Fig. 3, gm/gm product d).

**Rab GGTase Enzymatic Activity.** Rab GGTase activity in gm/gm and +/+ platelets was examined with an in vitro assay of Rabla prenylation (Fig. 4) (9). No other enzyme (including GGTase I and farnesyl-transferase) can prenylate Rab1a (14, 15). gm/gm Rab GGTase activity was reduced ~70% compared with +/+ platelets (Fig. 4), matching the decrease in Rabggtb protein. Similar results were reproducibly obtained with liver, kidney, and spleen extracts (Fig. 4 and data not shown). The gm reduction in Rab GGTase was specific: no difference was observed between gm/gm and +/+ in activity of GGTase-I, a related but different enzyme (16) (Fig. 4). Thus the Rabggtb splicing defect in gm results in decreased Rabggtb and Rab GGTase activity.

**Rab27 Hypoprenylation.** A previous study showed that gel migration of some small GTP-binding proteins was abnormal in gm platelets (2). Novel bands (~25 and ~28.5 kDa) of unknown identity were observed on GTP overlays of Western blots of gm platelets (Fig. 5) (2). These were not observed in gm liver, kidney, brain, spleen, macrophages or neutrophils (2). Rab proteins represented candidates for these abnormal GTP bind-
ing proteins. Specifically, Rab27a or Rab27b were excellent candidates because they are abundant in platelets (17). Blots stained with an antibody specific for both Rab27 isoforms disclosed a single band of 27.5 kDa in normal platelets and bone marrow, but an additional, mobility-shifted (28.5 kDa) isoform was observed in platelets (Fig. 5). Preliminary analyses suggest, as expected, deficiencies in prenylation of other Rabs in gm platelets (data not shown).

**Correlation of gm Genotype and Phenotype.** Evidence has been presented that a mutation in Rabggta, the gm genotype, results in impaired prenylation of Rab GGTase substrates, such as Rab27. Unprenylated Rab proteins lack the ability to attach to membranes and may account for the hematologic phenotype of gm (2, 3, 9, 12, 23). Because Rab proteins regulate intracellular protein trafficking and vesicular transport by cycling between membrane-bounded and cytosolic forms, failure to attach to intracellular membranes ablates this regulatory activity. Previous studies have documented disordered vesicular transport in gm platelets and megakaryocytes: Proteins destined for delivery from the trans-Golgi network to α-granules are misrouted in gm platelets (2, 3, 12). As a result, gm platelet α-granules lack fibrinogen, von Willebrand factor (vWF) and platelet factor 4. vWF is secreted from gm megakaryocytes instead of being transported to α-granules (2). In addition to these qualitative trafficking defects in α- and δ-granules, platelet synthesis is decreased in gm mice. Blood platelets are synthesized at the ends of proplatelet processes, long tube-like extensions produced by megakaryocytes (24). During platelet assembly the megakaryocyte demarcation membrane system is extensively and dynamically remodeled within the proplatelet processes. Further, platelet production within proplatelet processes critically depends

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**Fig. 3.** A splice acceptor mutation in Rabggta causes abnormal splicing. (A) Genomic structure of the 5′ end of mouse Rabggta. Exon α encodes 5′ untranslated region. The initiation methionine (bold and underlined ATG) occurs at the 3′ end of exon 1. Rabggta nucleotide sequence above is C57BL/6J-/+ and below is C57BL/6J-gm/gm. The splice acceptor mutation is indicated by *.* Rabggta exons connected by solid green lines represent normal splicing. Dashed red lines indicate exon 1 skipping in gm/gm RNA, and dotted blue lines indicate cryptic splice sites used by gm/gm. (B) RT-PCR analysis of RNA from wild-type (+/+ ) and mutant (gm/gm) bone marrow. The Rabggta oligonucleotides used amplified the region between exon α and exon 2. Each band was excised and sequenced: Normal bone marrow (+/+ ) shows the expected product of 486 bp (band a) (GenBank accession no. AF127656), and a product (638 bp) retaining intron α (band b) (GenBank accession no. AF127658). gm/gm bone marrow shows three bands: Band c (429 bp) (GenBank accession no. AF127657) represents exon 1 skipping; band b′ (638 bp) (GenBank accession no. AF127659) retains intron α; band d (557 bp) (GenBank accession no. AF127662) represents utilization of cryptic splice sites in intron α and exon 1. (C) Sequence analysis of Rabggta genomic DNA. Arrows indicate the splice acceptor mutation: +/+ = G, gm/gm = A, gm/+ exhibits both nucleotides (G and A).
Deficient prenylation of Rab27 in gm platelets. (A) Western blots of wild-type (+/+), heterozygous (gm/−), and mutant (gm/gm) platelets incubated with Rab27 and β-actin antibodies. Rab27 (60 kDa) was reduced ~70% in gm/gm. This experiment was repeated four times with the same results. (B) Rab GGTase activity of gm/gm and +/+ platelets. Duplicate cytosolic protein extracts from two samples of pooled platelets from gm (open symbols) and C57BL/6j mice (solid symbols) were assayed for Rab GGTase activity by measuring transfer of [3H]geranylgeranyl ([3H]GG) to Rab1a (15). This experiment was repeated four times with platelets, and twice with liver, spleen, and kidney protein extracts with similar results. (C) GGTase-I activity of gm/gm and +/+ pooled platelets. GGTase-I activity was determined by measuring transfer of [3H]GDP to Rac1. Each point represents an average of duplicate reactions. For Rab GGTase reactions, a control reaction incubated after addition of 50 mM EDTA was subtracted from all samples. For GGTase-I reactions, a control reaction incubated with buffer alone was subtracted from all samples. These experiments were repeated at least twice in each of the liver, kidney, and spleen extracts with similar results to platelets.

on both microtubule and actin cytoskeletal components (24). Potential roles of Rab proteins in platelet biogenesis are thus suggested. First, Rab proteins are well known to play key roles in membrane remodeling and trafficking (25). Second, several lines of evidence link Rab-mediated processes of vesicle trafficking with the actin and microtubule-based cytoskeletons (25). Finally, the distribution of membrane complexes is abnormal in gm megakaryocytes, and gm platelets are larger and more heterogeneous in size than normal (2). Thus, phenotypic effects of gm in bone marrow are explicable on the basis of Rab27 mutation.

Association of Rab GGTase with maintenance of normal platelet count and bleeding time implies that Rab GGTase represents a novel therapeutic target for thrombocytosis and clotting disorders (such as myocardial infarction, stroke, or deep venous thrombosis). Compounds that selectively inhibit Rab GGTase may be effective in these disorders when administered acutely to mimic gm by reducing platelet Rab GGTase activity.

Comparison of gm and Choroideremia (CHM). The molecular pathology, but not the phenotype, of gm resembles the X-linked human disorder CHM. In both, mutated genes encoding proteins involved in Rab geranylgeranylation result in Rab dysfunction: Rab27a is mutated in gm and REP1 is mutated in CHM (19, 20). Some Rab GGTase activity is retained in gm, probably by aberrant splicing that rescues certain Rab27a transcripts. In CHM, partial activity remains because two redundant loci involved in Rab geranylgeranylation result in Rab dysfunction: Rab27a and REP1 is mutated in CHM (19, 20). This experiment was repeated five times with the same results. (B) Western blots of subcellular fractions of wild-type (+/+), and mutant (gm/gm) platelets incubated with radiolabeled GTP or Rab27 antibodies. The subcellular fractions were total protein (T), membrane-associated protein (M), and soluble-fraction protein (S). This experiment was repeated five times with similar results. (C) Photomicrographs (×200) of +/+ and gm/gm platelets stained with Rab27 antibodies and detected with a FITC-conjugated secondary antibody. This experiment was repeated three times with similar results.

Human Platelet SPD Diseases. The human genetic disorders most similar to gm are GPS and α/δ-SPD (26). Like gm, GPS platelets are reduced in number, are increased in size, and lack α-granule proteins (26–28). Mis-sorting and inappropriate secretion of α-granule proteins occurs in GPS, as in gm (28). GPS patients, however, do not exhibit δ-granule storage pool deficiency or partial albinism. Like gm, α/δ-SPD is characterized by reduction in the number and contents of both α- and δ-granules (29). RABGGTA and RABGGTB are excellent candidate genes for GPS and α/δ-SPD.

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