Inherited Abnormalities in Platelet Organelles and Platelet Formation and Associated Altered Expression of Low Molecular Weight Guanosine Triphosphate-Binding Proteins in the Mouse Pigment Mutant Gunmetal


Gunmetal (gm/gm) is a recessively inherited mouse pigment dilution mutant that has high mortality and poor reproductive rates. In these studies, several hematologic defects were found associated with the mutation, including prolonged bleeding times, together with thrombocytopenia and increased platelet size. A unique feature is the presence of simultaneous abnormalities in two platelet organelles, dense granules and α-granules. The dense granule component serotonin is present at about half the normal concentration, as are visible dense granules. Three α-granule components (fibrinogen, platelet factor 4, and von Willebrand factor) are also significantly reduced. Thus, in several respects the gunmetal mutant resembles the human gray platelet syndrome. A novel abnormality in expression of low-molecular weight guanosine triphosphate (GTP)-binding proteins occurs in platelets of gunmetal. In Western blot assays, two additional GTP-binding proteins of 28.5 and 25 Kd were detected. The abnormal expression of GTP-binding proteins is, like the hematologic defects, genetically recessive and is tissue specific. Liver, kidney, brain, spleen, macrophages, and neutrophils have normal GTP-binding protein expression. The additional GTP-binding proteins are soluble. The data indicate that platelet formation and platelet organelle biogenesis are under common genetic control and that abnormal regulation of GTP-binding proteins may affect one or both processes.

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Submitted September 17, 1992; accepted December 23, 1992.

Supported by Grant No. HL 31698 from the National Heart, Lung and Blood Institute.

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0006-4971/93/8110-0021$3.00/0

MATERIALS AND METHODS

Animals. Gunmetal (gm/gm) mice, maintained on the B6C3Fe stock background, and other mutant mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and subsequently bred at the animal care facilities of the Roswell Park Cancer Institute. The gunmetal mutants were maintained by breeding heterozygous gm/+ mice by homozygous gm/gm mice.

Bleeding times. Bleeding time was determined by tail bleeding as described.22 Platelet collections and counts. Platelets were collected for serotonin determinations, thrombin-stimulated secretion studies, and Western blotting as described.28 Platelets were counted (3,000/analysis) and sized in citrated whole blood in a Coulter Model S+4 analyzer (Hialeah, FL) with channels set so that all particles between 2.0 and 20 μm³ were counted as platelets.

Platelet serotonin assay. Platelets were lysed in 1 mL distilled water and assayed fluorimetrically for serotonin according to Crosti and Lucchelli.29
Electron microscopy. Platelet-rich plasma was collected from citrated, undiluted blood by centrifugation at 150g for 10 minutes. Unfixed and unstained platelets were rapidly air-dried on carbon-coated grids. The number of dense granules larger than 50 nm was counted in individual platelets as described.

Thrombin-stimulated platelet secretion. To platelet-rich plasma, 0.5 µCi 14C serotonin (Amersham Corp, Arlington Heights, IL) was added and the mixture was allowed to incubate for 30 minutes at 37°C. Platelets were washed twice with phosphate-buffered saline, pH 7.4, containing 2% bovine serum albumin. Platelets were resuspended at 1 × 10^9/mL in 1.0 mL Tyrode’s solution (minus calcium plus magnesium). Thrombin, at various concentrations in 0.1 mL, was added and the mixture was allowed to incubate for 30 minutes at 37°C. Platelets were washed twice with phosphate-buffered saline, pH 7.4, containing 2% bovine serum albumin. Platelets were resuspended at 1 × 10^9/mL in 1.0 mL Tyrode’s solution. The pellet and supernatant were made 1% Triton X-100. Aliquots were assayed for radioactivity and lysosomal enzyme activity. β-Glucuronidase and β-galactosidase were assayed with fluorescein methyllumebilleryl substrates.

Lysosomal enzyme concentrations in kidney and liver extracts of testosterone-treated female mice were assayed as described.

Western blot assays of platelet factor 4 (PF4) fibrogenin, von Willebrand factor (vWF), and ras-related proteins. Extracts of platelets (10 to 60 µg protein) were electrophoresed on denaturing sodium dodecyl sulfate (SDS) acrylamide gels under reducing conditions as described. In both these assays and the detection of GTP-binding proteins described below, a proteinase inhibitor cocktail consisting of (final concentration) 1 mmol/L EDTA, 1 µg/mL chymostatin, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 5 µg/mL pepstatin, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) was included. The proteinase inhibitors were added during platelet collection and subsequently maintained in all platelet extract solutions. PF4 was quantitated on Western blots using a rabbit polyclonal antibody to rat PF4 at 200:1 dilution, followed by 125I-labeled goat antirabbit secondary antibody (ICN Biomedicals, Inc, Irvine, CA) at 1:100,001 dilution (0.4 aCi/mL). Radioactivity in PF4 components was determined at two quantities of platelet protein that differed by twofold, by storage phosphor technology with a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The storage phosphor screens store energy transferred from radioactive bands of Western blots. The exposed phosphor screens are, in turn, read in the PhosphorImager, where they are scanned with a focused HeNe laser to release the stored energy. The blue light emitted is collected and measured for each pixel. In comparison to traditional x-ray film autoradiography, storage phosphor screen autoradiography is faster and quantitatively linear over a wider dynamic range. Radioactivity in specific PF4 bands was corrected for background radioactivity in a region of the Western blot having no visible labeled bands. In some cases, a peroxidase-coupled goat antirabbit secondary antibody (Kirkegaard and Perry, Gaithersburg, MD) was used at 1:1000:1 to increase the sensitivity of detection of PF4.

In a similar fashion, fibrinogen and vWF were assayed in normal and mutant platelets. Rabbit antiserum to human fibrinogen was from Diagnostica Stago (Asnieres, France) and was used at 500:1 dilution in Western blots. Rabbit antiserum to human factor VIII (FVIII)-related antigen was from Accurate Chemical and Scientific Corp (Westbury, NY) and was used at 500:1 dilution.

Western blotting procedures were also performed using antibodies to human C-H ras (Oncogene Science, Inc, Manhasset, NY) to rat V-H ras (a gift of Dr G. Anderson, Roswell Park Cancer Institute, Buffalo, NY) and to peptide 57-76 of rat ras-4 (a gift of Dr J. Lenhard, Washington University, St Louis, MO).

Plasma vWF assay. Plasma vWF was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA) procedure using 1:500 rabbit antihuman vWF (Dako Corp, Carpenteria, CA) Ig fraction as the coating antibody and 1:4,000 peroxidase-conjugated anti-vWF (Dako Corp) as the detecting antibody, according to manufacturer’s instructions. ELISA reaction products were quantitated at 405 nm on a Bio-Tek EL309 microplate autoreader (Bio-Tek Instruments, Highland Park, VT).

Detection of GTP-binding proteins. Extracts of platelets and other tissues (30 to 70 µg protein) in the above described proteinase inhibitor solutions were electrophoresed on 12% denaturing SDS gels and blotted to nitrocellulose essentially as described by Lapeña and Reep. Use of freshly prepared mercaptoethanol in the SDS solution used to denature the platelet proteins, before the first electrophoresis, reduced variability in resolution of GTP-binding proteins. A modification was that 20% methanol and 0.1% SDS was included in the transfer buffer and [32P]GTP was increased to 2 µCi/mL during incubation of Western blots. Binding of GTP to specific platelet proteins was detected by incubation of blots with [α-32P]GTP followed by autoradiography.

To assay GTP-binding proteins in membrane and soluble fractions, platelets at 0.3 × 10^8/mL in hypotonic buffer (5 mmol/L Tris-Cl, 5 mmol/L EDTA, pH 7.5) were sonicated for 10 seconds at setting 1.5 in a Heat Systems Ultrasonic Processor W-220 (Heat Systems Ultrasonics, Inc, Farmingdale, NY) at 0°C. Sonicated extracts were centrifuged at 1,000g for 10 minutes to remove unbroken cells and debris. The supernatant was then centrifuged at 100,000g for 1 hour. This pellet was resuspended in the original volume of starting buffer and taken as the membrane fraction, and the supernatant was used as the soluble fraction. Extracts of whole platelets, membrane, and soluble fractions derived from an equivalent number of platelets were electrophoresed, blotted, and incubated with [32P]GTP.

RESULTS

Abnormal hemostasis in gunmetal mice. Prolonged bleeding times were found in all gm/gm mice tested (Table 1). In 11 gm/gm mice, bleeding times were greater than 15 minutes; in one other, bleeding time was prolonged to 8 minutes. In contrast, bleeding times in all seven gm/+ mice were normal (less than 5 minutes), thus establishing the recessive nature of the bleeding abnormality. Bleeding times were normal in a second recessive pigment dilution mutant, slaty (sl). When platelet number and size were determined (Table 1), it was apparent that gm/gm mice have both thrombocytopenia and somewhat increased platelet size. Platelet counts of gm/gm mice were approximately 50% those of gm/+ and mean platelet volumes of gm/gm were 30% larger. It is likely the mean platelet volume of 6.7 fl in gunmetal represents a minimum estimate because of the difficulty of distinguishing large platelets from small erythrocytes in whole blood analyses. This platelet size difference was maintained in gunmetal mice over a wide age span (from 4 weeks to 6 months).

Further analyses of peripheral blood of mutant mice showed no other significant quantitative abnormalities for total white or red blood cell numbers or hemoglobin concentrations in gunmetal. Also, microscopic analysis of blood smears stained with Wright's stain showed no significant qualitative abnormalities of blood cells in the gm/gm mutant as compared with gm/+. Dohle bodies, similar to those characteristic of the May-Hegglin anomaly, were not observed in.

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Table 1. Prolonged Bleeding Time and Platelet Abnormalities in Gunmetal Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bleeding Time [min]</th>
<th>Platelet Count [x10^9/µL]</th>
<th>Mean Platelet Volume (FL)</th>
<th>Serotonin (μg/10^9) Platelets</th>
<th>Dense Granules/Platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td>gm/+</td>
<td>1.4 ± 0.09 (7)</td>
<td>913 ± 50 (9)</td>
<td>5.1 ± 0.12 (9)</td>
<td>3.02 ± 0.23 (7)</td>
<td>9.52 ± 0.90 (50)</td>
</tr>
<tr>
<td>gm/gm</td>
<td>&gt;15 (11)*</td>
<td>468 ± 36 (6)*</td>
<td>6.7 ± 0.32 (3)*</td>
<td>1.43 ± 0.23 (7)*</td>
<td>4.59 ± 0.52 (61)*</td>
</tr>
<tr>
<td>slt/+</td>
<td>2.4 ± 0.48 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>slt/slt</td>
<td>2.3 ± 0.22 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM of the number of mice in parentheses (except for dense granules/platelet, in which the values in parentheses are the number of individual platelets analyzed). Values for ++/+ mice are not included, as no significant differences were noted between gm/+ and +/+ mice in the above measurements.

* P < .001.
† P < .01.
‡ P < .0002.

Peripheral blood granulocytes of gm/gm mice (Dr Judith Wall, personal communication, 1992).

Dense granule abnormalities. By two criteria, gm/gm mice exhibit a mild SPD (Table 1). First, the concentration of serotonin, a typical dense granule constituent, is reduced to less than half of the control values. Second, the number of dense granules per platelet detectable after whole mount electron microscopy is similarly reduced to about 50% of control values. A comparative distribution of morphologically observable dense granules in platelets of gm/gm and gm/+ is given in Fig 1. In gm/+ platelets, the mean number of dense granules per platelet is 9 to 10, with rare platelets having as few as 0 or as many as 30 granules per platelet. In contrast, the distribution in gm/gm is skewed toward fewer granules per platelet. In fact, 14 of 61 platelets had no observable dense granules. Because gunmetal platelets are, on average, larger than normal platelets (Table 1), it follows that the dense granule deficiency, per platelet volume, is larger than that indicated in Fig 1. Because of the larger volume of gunmetal platelets, the measured dense granule deficiencies are minimal estimates.

α-Granule abnormalities. Western blotting procedures were used to enumerate platelet α-alpha granule proteins. Specific immunoiodolabeled components were quantitated on blots by storage phosphor technology. Platelet fibrinogen was detected as a set of three bands between 57 and 66 Kd (Fig 2A). The intensity of these radiolabeled components was equivalent in ++/+ and gm/+ platelets and was reduced in gm/gm platelets, indicating that the reduction of fibrinogen, like the pigment dilution in gunmetal, is genetically recessive. An additional band of unknown origin at 38 Kd was present at equal intensity in all samples.

Similar results were obtained in analyses of platelet vWF (Fig 2B). A major band at 240 Kd was present at equal quantities in ++/+ and gm/+ platelets and was reduced in gm/gm platelets.

Immunoblots for PF4 showed three components of expected LMW. Two minor bands appeared at 16 and 15 Kd and a major form appeared at 13 Kd (Fig 2C). Platelets from mice homozygous for gunmetal showed a considerable reduction specifically in these LMW bands. The intensities of these bands were similar in gm/+ and ++/+ mice. There were also several additional bands of unknown origin at higher molecular weight that were present at equal intensity in mice of all three genotypes. These are likely nonspecific components that appeared because of the low cross-reactivity of the antibody to rat PF4 with mouse PF4 and the subsequent necessity of using ultrasensitive detection procedures. Because the nonspecific components were sufficiently removed from the specific LMW PF4 bands, they did not interfere with the analyses.

When specific fibrinogen, vWF, and PF4 components were quantitated by phosphor storage technology (Table 2), a general reduction of all three components to 40% to 50% of

![Fig 1. Distributions of dense granules in platelets from (A) normal (gm/+ ) and (B) gunmetal (gm/gm) mice. Platelets were air-dried, and unstained whole platelets were examined for dense granules by electron microscopy. The number of platelets analyzed was 50 (gm/+ ) and 61 (gm/gm) from three mice each. Values for (+/+ ) platelets are not included as no significant differences were noted between gm/+ and +/+ .]
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Fig 2. Reduced concentrations of α-granule components in platelets of gunmetal. Equal amounts of protein from platelet extracts of three separate +/+ , gm/+ , and gm/gm mice were electrophoresed and Western blotted. Nitrocellulose membranes were treated with antibody to (A) fibrinogen, (B) vWF, or (C) PF4. In the fibrinogen and vWF analyses, this was followed by treatment with [3H]-labeled secondary antibody and autoradiography. In PF4 analyses, peroxidase-labeled secondary antibody was used for increased sensitivity. The PF4 components are indicated by a bracket.

normal levels was observed. Also, in all three cases, the reduction was genetically recessive, ie, mice heterozygous for the gunmetal gene had normal concentrations.

Plasma of gunmetal mice was analyzed to determine if there was an increase in plasma vWF concomitant with the platelet vWF reduction. Indeed, gm/gm plasma vWF units (154 ± 10 U/dL) were significantly (P < .02) increased relative to gm/+ plasma units (91 ± 12 U/dL).

Normal lysosomal enzyme concentrations and thrombin-stimulated secretion. No abnormalities were detected in another platelet subcellular organelle, the lysosome. Concentrations of two lysosomal enzymes, β-glucuronidase and β-galactosidase, were normal in gm/gm mice (Table 3).

In the case of several other mouse pigment mutants with SPD,1,2,4,5 there is an associated increase in concentrations of kidney lysosomal enzymes due to a lowered rate of secretion of kidney lysosomal enzymes into urine. However, kidney glucuronidase and galactosidase concentrations were normal in gm/gm mice, as were concentrations of these two enzymes in a control tissue, liver (Table 3). Likewise, measurements of thrombin-stimulated secretion from platelets of the dense granule component serotonin and two lysosomal enzymes showed only slight decreases in the secretion of serotonin and glucuronidase from gunmetal platelets (Table 4).

Altered expression of GTP-binding proteins in gunmetal platelets. Several LMW (27.5, 26, and 23 Kd) GTP-binding proteins were detected (Fig 3) in normal (+/+ ) platelets when Western blots of platelet extracts were probed with [35P]GTP. The pattern of expression in homozygous gunmetal (gm/gm)
was quite different in that at least two additional LMW GTP-binding proteins were apparent, one of higher molecular weight (28.5 Kd) and another of 25 Kd. In addition, all GTP-binding proteins expressed in normal platelets were expressed in gunmetal platelets. The degree of resolution of the normal 23-Kd GTP-binding proteins was variable in both normal and mutant mice. Recent experiments have shown that the degree of resolution of GTP-binding proteins is sensitive to the reducing environment during SDS electrophoresis. Use of freshly prepared mercaptoethanol and/or dithiothreitol reduces this variability. The 28.5- and 25-Kd GTP-binding proteins were consistently observed in platelets from 14 separate gm/gm mice and were never observed in platelets from 12 gm/+ mice. No difference in expression of platelet LMW GTP-binding proteins was observed between male and female mice.

The proteins detected by this assay are GTP specific. Inclusion of 10 µmol/L GTP or GTPyS in the [32P]GTP incubation buffer resulted in no detectable radiolabeled bands in either +/- or gm/gm platelet extracts, an effect not observed with 10 µmol/L adenosine triphosphate (ATP) or guanosine monophosphate (GMP) (not shown). In fact, increased [32P]GTP-binding to all proteins was observed in the presence of ATP or GMP.

The total amount of LMW GTP-binding proteins in gunmetal platelets, as detected by densitometric scans of Western blots, is increased 2.1-fold over that of normal platelets. The increased expression in gunmetal platelets is entirely due to expression of the new 28.5- and 25-Kd GTP-binding proteins. Expression of the remaining GTP-binding proteins is equal in normal and gunmetal platelets.

The expression of the additional LMW GTP-binding proteins is genetically recessive. The pattern in platelets from heterozygous (gm+) mice is identical to that of normal (+/+) platelet (Fig 3). This result is in agreement with the recessive nature of other characteristics in gm/gm mice.

The abnormal expression of gunmetal GTP-binding proteins is specific to platelets. Western blots of extracts from brain, kidney, spleen, and bone marrow (Fig 4) showed tissue-

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**Table 2. Reduction of α-Granule Components in gm/gm Platelets**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Relative Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrogen</td>
<td>vWF</td>
</tr>
<tr>
<td>+/-</td>
<td>664 ± 43</td>
</tr>
<tr>
<td>gm/+</td>
<td>647 ± 32</td>
</tr>
<tr>
<td>gm/gm</td>
<td>266 ± 31*</td>
</tr>
</tbody>
</table>

Platelet extracts (50 µg protein) were electrophoresed, transferred to nitrocellulose membranes by Western blotting, and treated with antibodies to either fibrinogen, vWF, or PF4 followed by [35S]labeled second antibody. Relative units of radioactivity in specific fibrinogen, vWF, or PF4 bands were determined by phosphor storage technology. Results are expressed as the mean ± SEM of determinations on extracts from three mice of each genotype.

* P ≤ .01.
† P ≤ .02.

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**Table 3. Concentrations of Lysosomal Enzymes in Kidney, Liver, and Platelets of gm/+ Normal and gm/gm Mutant Mice**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Genotype</th>
<th>β-Glucuronidase</th>
<th>β-Galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>gm/+</td>
<td>118 ± 17</td>
<td>25.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>gm/gm</td>
<td>112 ± 6.7</td>
<td>18.0 ± 2.7</td>
</tr>
<tr>
<td>Liver</td>
<td>gm/+</td>
<td>22.0 ± 0.67</td>
<td>9.58 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>gm/gm</td>
<td>22.4 ± 1.1</td>
<td>10.1 ± 0.39</td>
</tr>
<tr>
<td>Platelets</td>
<td>gm/+</td>
<td>0.0437 ± 0.0028 (8)</td>
<td>0.0745 ± 0.0023 (8)</td>
</tr>
<tr>
<td></td>
<td>gm/gm</td>
<td>0.0423 ± 0.0040 (7)</td>
<td>0.0656 ± 0.0050 (7)</td>
</tr>
</tbody>
</table>

Kidney and liver tissues were from mice treated with testosterone as described in Materials and Methods. Platelets were from untreated male or female mice. Values are the mean ± SEM of the number of mice indicated in parentheses. Values for kidney and liver are units per gram of tissue. Values for platelets are units per milligram of protein.
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**Table 4. Thrombin-Stimulated Secretion of Platelet Dense Granule Contents and Lysosomal Enzymes**

<table>
<thead>
<tr>
<th>Thrombin (UI)</th>
<th>0</th>
<th>0.05</th>
<th>0.25</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>14C-serotonin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gm/+</td>
<td>8.7 ± 2.4</td>
<td>33 ± 1.7</td>
<td>80 ± 3.0</td>
<td>88 ± 1.7</td>
</tr>
<tr>
<td>gm/gm</td>
<td>11 ± 1.8</td>
<td>26 ± 8.2</td>
<td>57 ± 3.8*</td>
<td>72 ± 0.9t</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gm/+</td>
<td>8.3 ± 3.8</td>
<td>8 ± 3.8</td>
<td>20 ± 0.33</td>
<td>29.0 ± 2.5</td>
</tr>
<tr>
<td>gm/gm</td>
<td>9.3 ± 2.2</td>
<td>9.3 ± 2.2</td>
<td>15 ± 1.5t</td>
<td>23.3 ± 1.5</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gm/+</td>
<td>5.7 ± 2.2</td>
<td>11 ± 4.4</td>
<td>25 ± 4.2</td>
<td>36 ± 3.2</td>
</tr>
<tr>
<td>gm/gm</td>
<td>4.6 ± 0.88</td>
<td>12 ± 2.0</td>
<td>21 ± 1.5</td>
<td>30 ± 3.8</td>
</tr>
</tbody>
</table>

Total cpm [14C]serotonin initially incorporated in gm/+ and gm/gm platelets were 10,700 and 10,400, respectively, per 10⁸ platelets. Values are the mean percentage secretion ± SEM of measurements on platelets from three experiments with three mice in each experiment.

* P < .001.
† P < .01.
‡ P < .05.

...specific patterns of expression. However, each tissue pattern was equivalent in gunmetal and normal extracts. In other experiments (not shown), equivalent GTP-binding protein expression was observed in extracts from normal and gunmetal liver, neutrophils, macrophages, and retinal pigment epithelium.

Because platelet SPD was found in gunmetal, platelet extracts of other pigment mutant mice previously shown to have SPD[1–5] were tested for abnormal expression of GTP-binding proteins (Fig 5). A normal GTP-binding protein pattern was apparent in the pallid, pale ear, muted, ruby-eye-2, pearl, light ear, and beige mutants. In other experiments (not shown), normal GTP-binding protein expression was also observed in platelet extracts of other mouse pigment mutants with platelet SPD, including the cocoa, mocha, muted, sandy, and ruby-eye mutants.

A hallmark of many LMW GTP-binding proteins is that they are located at specific subcellular membrane sites.[25,26] In fact, the GTP-binding proteins of normal (gm/+ ) platelets are predominantly located in the membrane fraction (Fig 6). The 28.5- and 25-Kd GTP-binding proteins specifically expressed in gunmetal platelets are, on the other hand, concentrated in the soluble fraction (Fig 6). In some cases, the subcellular locations of GTP-binding proteins change in response to specific external signals.[37] However, treatment of gunmetal or normal platelets with the potent agonist thrombin (2.5 U/mL for 3 minutes) did not cause a shift of the abnormal GTP-binding proteins of gunmetal to the membrane fraction, nor in fact did it affect the subcellular distribution of any GTP-binding protein of either gunmetal or normal platelets (not shown).

**DISCUSSION**

The hemorrhagic abnormalities of the gunmetal mutant are unusual in that they include not only prolonged bleeding but also apparent widespread abnormalities in both platelet formation and in platelet organelle biogenesis. Abnormalities in platelet formation are suggested by the lowered platelet count and an accompanying mild increase in platelet volume. It is possible that the increase in platelet volume is a result of accelerated platelet production in response to the thrombocytopenia. Associated with this macrothrombocytopenia are abnormalities in at least two organelles. Dense granules and their serotonin content are approximately halved and the α-granule components fibrinogen, vWF, and PF4 are at 40% to 50% of normal levels. Gunmetal is the first mutant mouse model reported to exhibit both dense granule and α-granule abnormalities.

The fact that several α-granule components are reduced in platelets of gunmetal indicates a general effect on organelle biogenesis or processing rather than on specific α-granule components. The finding that vWF that is absent in platelets is present as excess plasma vWF suggests an abnormality in targeting or retention of α-granule components rather than a defect in synthesis. No morphologic abnormalities of gunmetal platelets have been noted at the electron microscope.
level other than a reduced number of α-granules (R.T.S., unpublished observations) and the described reduction in dense granule number.

Gunmetal has some similarities to several inherited human hemorrhagic diseases. It is similar to Hermansky-Pudlak syndrome (HPS)\textsuperscript{13,14} in that they both have pigment dilution and SPD. However, they differ in that the SPD is more severe in HPS\textsuperscript{13,14} in that both have pigment dilution and hemorrhagic diseases. It is similar to Hermansky-Pudlak and Bernard-Soulier syndrome\textsuperscript{39} patients have, like gunmetal, granule components. The human May-Hegglin anomaly\textsuperscript{31} of the gunmetal phenotype to human SPD\textsuperscript{13,14} in that both have deficiencies of α-granules and dense granules.

Among inherited human bleeding diseases, gunmetal is most similar to the human gray platelet syndrome\textsuperscript{23-25} in that both have deficiencies of platelet α-granule proteins, reduced platelet numbers, and enlarged platelet volumes. The appearance of platelet α-granule proteins in plasma is likewise common to both. Gray platelet patients usually have a more marked deficiency of α-granule components and do not exhibit SPD. However, the SPD difference may be more apparent than real because SPD in gunmetal is mild (twofold decrease in dense granule contents) and because a similar twofold variation in platelet dense granule contents is found among human controls.\textsuperscript{22}

In several respects, the hemostatic abnormalities of gunmetal resemble those recently reported for the Wistar-Furth rat.\textsuperscript{40-42} Both have macrothrombocytopenia accompanied by partial deficiencies in α-granule components that are inherited recessively. Another interesting similarity is that both have lowered reproductive rates. A difference is that the size of gunmetal platelets does not increase with age as has been found for the Wistar-Furth rat.

Gunmetal represents the twelfth mouse mutant with combined pigment dilution, prolonged bleeding, and platelet SPD symptoms.\textsuperscript{1,16} Thus far, 13 of 36 pigment dilution mutants surveyed have SPD. However, the abnormalities in hemostasis in gunmetal are quite distinct and more widespread in nature as compared with the other mouse pigment mutants. It is the only one with either decreased platelet number or increased platelet volume. The SPD is milder in gunmetal. In all other mouse SPD mutants, the highest concentrations of dense granule components or of morphologically detectable dense granules are 20% of normal or less, whereas dense granule components in gunmetal platelets are 50% of normal. Another difference is that deficiencies of α-granule components have not been detected in other mouse mutants with SPD (R.T.S., unpublished observations). Finally, gunmetal is the only mouse hemorrhagic mutant with abnormal expression of LMW GTP-binding proteins.

LMW GTP-binding proteins comprise a very large multigene superfamily, including the ras, rho, rab, ran, and arf subfamilies.\textsuperscript{43} They have been implicated in the regulation of intracellular vesicle traffic and function.\textsuperscript{25,26,44-48} Individual GTP-binding proteins are often localized to specific subcellular organelles and are thought to impart specificity to the membrane budding and fusion reactions involved in intracellular vesicle traffic, thereby ensuring accurate delivery of transport vesicles to their correct targets. Although the biochemical and molecular properties of LMW GTP-binding proteins have been well studied, their organelar function(s), in mammalian systems and particularly in platelets,\textsuperscript{49} are little understood.

The altered expression of GTP-binding proteins in gunmetal platelets is not likely due to simple redistribution of pre-existing normal GTP-binding proteins because "normal" GTP-binding proteins are present in gunmetal platelets at the same quantity as they are found in normal platelets. It appears that new GTP-binding proteins of 28.5 and 25 Kd are synthesized or otherwise expressed in gunmetal platelets. The normal allele at the gunmetal gene serves as a dominant suppressor of expression of the GTP-binding proteins because no abnormal expression of GTP-binding proteins is apparent in heterozygous mice. However, additional studies are required to determine if the product of the gunmetal gene directly regulates expression of GTP-binding proteins.

The identities of the new 28.5- and 25-Kd GTP-binding proteins expressed in gunmetal platelets are unknown. The possibilities are large because in platelets alone at least 15
LMW GTP-binding proteins have been identified. The 28.5- and 25-Kd GTP-binding proteins do not react to antibodies to C-H ras or V-H ras or rab-4 GTP-binding proteins in Western blots (R.T.S., unpublished observations).

The abnormal expression of GTP-binding proteins exhibits a striking tissue specificity. Among nine tissues tested, it was found only in platelets. The normal expression patterns in bone marrow and spleen are not indicative of normal expression in megakaryocyte precursors of platelets because abnormal GTP-binding proteins expression in a minor cell population would not be detected by these techniques. The normal expression in retinal pigment epithelium is consistent with normal eye color in gunmetal mutants. It will be interesting to determine if, reflective of the coat color dilution, GTP-binding protein expression is altered in skin melanocytes of gunmetal given the gene’s effect on coat color.

Because it has been established that LMW GTP-binding proteins are involved in organelar formation and function in other systems, it may be speculated that the abnormal expression of LMW GTP-binding proteins cause the dense granule and α-granule abnormalities in gunmetal mice. An abnormality in LMW GTP-binding proteins expression in...
megakaryocytes might result in abnormal biogenesis of platelet granules. However, these experiments establish that grossly abnormal expression of platelet LMW GTP-binding proteins is not obligatorily associated with SPD because 11 other mouse mutants with SPD have no obvious abnormalities in these proteins.

The abnormal GTP-binding proteins in gunmetal are not involved in the regulation of secretion of platelet organelles because there is little or no effects on secretion of dense granules or lysosomes in gunmetal. Also, the fact that the subcellular location of the abnormal GTP-binding proteins does not change in response to thrombin treatment is consistent with the notion that they do not regulate secretion. Physiologic stimuli cause changes of the subcellular location of GTP-binding proteins of the secretory pathway in other systems.

An alternative possibility is that the abnormal expression of GTP-binding proteins in gunmetal causes the abnormal platelet formation. Platelet formation from megakaryocytes includes extensive membrane budding and fusion. Therefore, it is reasonable to speculate that GTP-binding proteins are involved in platelet formation because in general they regulate membrane budding and fusion. The fact that the gunmetal GTP-binding protein abnormality is found only in platelets is consistent with a defect in a platelet-specific process, such as platelet formation. Also, this interpretation is consistent with the fact that, among all the 12 mouse mutants with platelet organellar abnormalities, only the gunmetal mutant, with its unique abnormalities in GTP-binding proteins, has an accompanying abnormality in platelet formation. It may be relevant that the GTP-binding protein CDC42 is involved in regulation of cell polarity and budding in yeast. Establishment of cell polarity in yeast appears to be mediated by the actin cytoskeleton. Evidence for a role of the megakaryocyte cytoskeleton in platelet formation has been presented. Analyses of GTP-binding protein regulation in gunmetal megakaryocytes will contribute to the resolution of this proposal.

The combined abnormalities in gunmetal suggest that formation of platelets and their organelles may be under common genetic control. The gunmetal mouse provides a model of hereditary macrothrombocytopenia that should be useful in studying the determination and regulation of platelet formation and/or organelle biogenesis during megakaryocyte differentiation and the role of GTP-binding proteins in these processes.

ACKNOWLEDGMENT

We thank Dr David J. Kuter of the Department of Biology of the Massachusetts Institute of Technology for the gift of rabbit antisera to rat platelet factor 4. Ed Hurley and Al Cairo analyzed platelets by electron microscopy, while John Mierzwa and Roger Palmer provided platelet counting and sizing. We thank Jie Wang for able technical assistance and Cheryl Mrowczynski and Cynthia Bates for expert secretarial assistance.

REFERENCES


20. Nieuwenhuis HK, Akkerman J-WN, Sixma JJ: Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency. Studies on one hundred six patients. Blood 70:620, 1987
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35. Lapetina EG, Reep BR: Specific binding of [α32P]GTP to cytosolic and membrane-bound proteins of human platelets correlates with the activation of phospholipase C. Proc Natl Acad Sci USA 84:2261, 1987


45. Segev N, Mulholland J, Botstein D: The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. Cell 52:915, 1988


53. Bender A, Pringle JR: Multicopy suppression of thecdc24 budding defect in yeast by CDC42 and three newly identified genes including the ras-related gene RSR1. Proc Natl Acad Sci USA 86:9976, 1989


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Inherited abnormalities in platelet organelles and platelet formation and associated altered expression of low molecular weight guanosine triphosphate-binding proteins in the mouse pigment mutant gunmetal

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