Circulating blood platelets regulate the initial phase of the hemostatic response through adhesive and aggregatory events and by providing the necessary procoagulant surface for prothrombinase complex assembly and thrombin generation. The signaling pathway(s) that regulate platelet procoagulant activity are largely unknown, although they are distinct from platelet aggregatory signals linked to fibrinogen ligation to the conformationally active $\alpha_{IIb}\beta_3$ integrin. We describe a novel intracellular signaling mechanism involving platelet IQGAP1 that specifically regulates the development of platelet procoagulant activity under conditions of mechanical shear stress. Murine platelets that are deficient in IQGAP1 demonstrate increased prothrombinase activity compared with wild-type littermate controls when activated by a physiological shear stress of 16 dynes/cm$^2$ (shear rates of 1600 s$^{-1}$ ($p < 0.0001$), corresponding to $\sim 2.5$ times the normal shear stress, or $\sim 40\%$ degree of stenosis in coronary arteries. The exaggerated prothrombinase activity is not associated with enhanced platelet microvesiculation (cytoskeletal proteolysis) and occurs independently of the intracellular calcium release, [Ca$^{2+}$], but it is specifically coupled to the $\alpha$-granule exocytic pathway without concomitant effects on aminophospholipid exposure. These observations identify platelet IQGAP1 as an important modulator of normal hemostasis and as an appropriate pharmacological target for control of platelet procoagulant function.

The hemostatically neutral surface of quiescent platelets is preserved by an aminophospholipid translocase that maintains phosphatidylserine (PS) and phosphatidylethanolamine within the inner platelet leaflet and phosphatidylcholine on the outer leaflet. This asymmetry is abolished by thrombin, collagen, and ionophores, presumably related to changes in the intracytosolic calcium concentration, [Ca$^{2+}$]. The rapid loss of membrane asymmetry is mediated by a calcium-dependent phospholipid scramblase (with inhibition of the translocase), resulting in rapid bidirectional movement of phospholipids, the appearance of procoagulant PS, and an $\sim 6$-log acceleration of $\alpha$-thrombin generation via the assembly of cell surface prothrombinase (1, 2). The importance of the sudden reversal of this membrane phospholipid in the maintenance of normal hemostasis is best exemplified by Scott’s syndrome. This syndrome is a rare bleeding disorder caused by an uncharacterized molecular defect associated with a platelet procoagulant deficiency, which is related to defective membrane PS movement (3). Nonetheless, the aggregatory potential of platelet agonists (mediated by fibrinogen ligation to the conformationally active $\alpha_{IIb}\beta_3$ integrin) is distinct from that linked to exposure of the aminophospholipids PS and phosphatidylethanolamine. In thrombin-activated human platelets, for example, PS exposure is mediated by protease-activated receptor-1 (PAR1), with little or no contribution from PAR4 (2, 4).

Platelet microvesiculation (generation of platelet microparticles (PMP)) is closely associated with the exposure of anionic PS on the outer surface of the platelet, thereby considerably enhancing the surface area for activation of the coagulation cascade. PMPs can be generated from high shear stress and are able to cross-activate platelets and endothelial cells, and they are elevated in patients with increased thromboembolic risk and in the subsets of those patients undergoing coronary artery bypass grafting (1, 2). A causal role for PMPs in thrombosis, however, has been difficult to establish because platelet microvesiculation is intimately associated with platelet activation and aggregation. Although the molecular basis of microvesiculation remains poorly characterized, it appears to be calcium-dependent, requires cytoskeletal reorganization, is associated with calpain and caspase-3 activation, and is morphologically similar to the membrane blebbing and phospholipid exposure phase of nucleated cell apoptosis (5). The molecular mechanism of PMP generation remains unknown, although the molecular pathway appears to be distinct from that regulating PS exposure or platelet exocytosis and is associated with cytoskeletal proteolysis (1–3).

Mammalian IQGAPs are conserved homologues of an extended family of proteins; their counterparts in yeast (Saccharomyces cerevisiae Iqg1p/Cyk1p) and amoebae (Dictyostelium discoideum DdGAP1) have key roles in actomyosin ring formation and cytokinesis. Two homologous IQGAPs have been identified and characterized, although expression of IQGAP1 appears broad compared with the previously described hepatocyte-restricted distribution of IQGAP2 (6, 7). IQGAPs are multidomain scaffolding proteins that presumably integrate intracellular signals with actin polymerization by interactions with the Rho GTPases rac1 and cdc42 (through Ras-GAP-related homology domains, or GRD), Ca$^{2+}$/calmodulin (through IQ motifs), and F-actin (through calponin homology domains). Inter-
actions with E-cadherin (8), β-catenin (9), and the microtubular protein CLIP-170 (10) have been described previously, although the in vivo physiological significance of these interactions and the regulatory mechanisms leading to IQGAP activation remain incompletely characterized. Our data now link IQGAP1 to a shear stress-restricted (mechanoreceptor) signaling pathway of platelet activation, specifically regulating the procoagulant properties of the platelet membrane.

MATERIALS AND METHODS

Murine Functional Studies—All animal studies were completed in accordance with approved Institutional Animal Care and Use Committee protocols at the State University of New York, Stony Brook. mIQGAP1+/− mice and inbred wild-type littermate controls were expanded and maintained on an SV129 background and genotyped by PCR (11). Blood was collected by retro-orbital puncture into 0.1 volume of acid/citrate/dextrose supplemented with 0.1 μM prostat glandulin E2, and gel-filtered platelets (GFP) were isolated over a Sepharose 2B column as described previously (12). Protein-solubilized lysates were generated from liquid nitrogen snap-frozen livers or GFP (1 × 10⁹ platelets), and immunoblot analysis was completed by 4–15% SDS-PAGE using anti-IQGAP1 (1:500 dilution) (Transduction Laboratories). IQGAP2 (1:10000) (Upstate Biotechnologies) antibodies, which were detected by enhanced chemiluminescence (Amersham Biosciences). Complete hemograms were determined using automated Coulter counters, and the F-actin content of GFP was determined by flow cytometry using fixed and permeabilized platelets incubated with 30 units/ml FITC-phallolidin (13). Transmission electron microscopy was completed using 1 × 10⁶ GFP fixed in 2.5% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde. After postfixation in a freshly prepared solution of 1% (w/v) osmic acid, alcohol-dehydrated samples were pelleted and embedded in an Epon-812 resin mixture, and thin sections were examined in a blinded fashion.

Platelet Functional Studies—Gel-filtered platelets in HEPES-buffered modified Tyrode’s (HBMT) solution (138 mM NaCl, 2.7 mM KCl, 0.1 mM NaH2PO4, 1 mM NaHCO3, 0.2% bovine serum albumin, 0.1% dextrose, and 10 mM HEPES, pH 7.4) were rested at 37 °C for 30 min prior to use and then supplemented with 80 μM human fibrinogen prior to aggregation (with stirring) using a 4-channel Chronolog (Hormon-Chemie), or ADP (Sigma). Microspectrofluorimetry was completed in 96-well microtiter plates using a FLEXStation (Molecular Devices). GFP (1 × 10⁵/well) were gently centrifuged (800 × g) into individual wells and loaded with Fluo-3 (Flex reagent, Molecular Probes, Eugene, OR) for 45 min at 37 °C followed by a second gel filtration step prior to shear activation (or microspectrofluorimetry using 1 mM α-thrombin). For some experiments, determining procoagulant activity of the microparticle fractions was carried out in parallel using the supernatants of samples centrifuged at 10,000 × g (flow cytometry using FITC-conjugated anti-CD41 confirmed the presence of <2% intact platelets in microparticle fractions). Control experiments on uncirculated platelets showed no detectable procoagulant activity.

RESULTS

IQGAP Characterization in Murine Platelets—We have previously identified both IQGAP1 and IQGAP2 in human platelets and demonstrated that IQGAP2 activation is restricted to platelet stimulation by thrombin (13). Targeted disruption of the mIQGAP1 gene has been described previously, with no evident phenotypic consequences except for gastric hyperplasia (11). Immunoblot analysis using either murine liver or platelet lysates established that murine platelets specifically express mIQGAP1, with no evidence for mIQGAP2 expression (Fig. 1). These expression patterns are distinct from those of human platelets, which express both IQGAP1 and IQGAP2. Furthermore, there are no compensatory changes in mIQGAP2 expression levels in mIQGAP1+/− platelets. Complete blood counts from 30 mice (wild type (n = 10)), mIQGAP1+/− heterozygotes (n = 9), and mIQGAP1−/− (n = 11) demonstrated no differences in hemoglobin, hematocrit, white blood cell counts, platelet counts, mean platelet volumes, platelet F-actin content, or morphology as evaluated by Wright-Giemsa staining or transmission electron microscopy. Similarly, in a comparison of heterozygote, wild-type controls (n = 3 in each arm), and mIQ
GAP1<sup>−/−</sup> platelets, platelet aggregometry using gel-filtered platelets demonstrated small but nonreproducible differences in agonist-induced hyperresponsiveness. These results were most evident using strong receptor agonists such as thrombin or fibrillar collagen (known to activate platelets via members of the PAR family or the glycoprotein VI/FcγR-chain receptor tyrosine kinase complex, respectively) and, when evident, were manifest by a more rapid initial slope, with slightly exaggerated amplitudes (data not shown).

**Exaggerated Calcium Mobilization in mIQGAP1<sup>−/−</sup> Platelets**—Because IQGAP1 is known to bind Ca<sup>2+</sup>/calmodulin via four homologous IQ motifs (IQXXRXQXXR) (17), microspectrofluorimetry was completed to establish the effect of IQGAP1 deficiency on intracellular calcium responses ([Ca<sup>2+</sup>]i) in agonist-stimulated platelets. Initial studies using gel-filtered platelets from mIQGAP1<sup>−/−</sup> mice demonstrated exaggerated elevations of intracytosolic calcium compared with wild-type controls at both concentrations of thrombin (1 and 10 nM). Subsequent experiments using both male and female mice (n = 3 in each arm) confirmed these results, extending these observations to platelet stimulation using the PAR4-activating peptide AYPGKF (Fig. 2). The exaggerated responses were readily evident in the rate of cytosolic calcium release and were concentration-dependent, using both thrombin and the PAR4-activating peptide. Because IQGAP1 is a major calmodulin-binding protein, we sought to exclude the possibility that these exaggerated [Ca<sup>2+</sup>]i responses were caused by secondary changes in cytosolic calmodulin concentrations. As seen in Fig. 2, calmodulin concentrations were essentially identical between mIQGAP1<sup>−/−</sup> and wild-type platelets and comparable with those previously determined in human platelets (19).

**Shear Stress-restricted Defect in mIQGAP1<sup>−/−</sup> Platelet Procoagulant Activity**—Platelet procoagulant activity is intimately associated with the calcium-induced microvesiculation that parallels the generation of the prothrombinase complex, although the two endpoints are independently regulated molecular events (20, 21). Thus, whereas microvesiculation requires cytoskeletal proteolysis, assembly of the prothrombinase complex is contingent on two processes: (i) the release of activated platelet factor V from the storage pool found within α-granules (see below), and (ii) exposure of aminophospholipids, including negatively charged PS. Different platelet agonists cause the extent of PS exposure to vary. For example, ionophores typically effect maximum exposure followed by fibrillar collagen and thrombin; PS exposure is also known to result from mechanical shear stress (22). To further dissect the relationship of the exaggerated [Ca<sup>2+</sup>]<sub>i</sub>, release in response to PS exposure, platelets were activated by distinct agonists or by mechanical shear, and procoagulant activity was determined using a modified prothrombinase assay that closely parallels PS exposure as quantified by annexin-V binding (15). Consistent with prior observations (2, 4), thrombin and collagen (but not ADP or PAR4 agonist peptide) caused dose-dependent increases in prothrombinase activity, results that were essentially the same for both mIQGAP1<sup>−/−</sup> and wild-type platelets (Fig. 3a). In sharp contrast, however, mIQGAP1<sup>−/−</sup> platelets activated by shear stress demonstrated enhanced prothrombinase activity compared with littermate controls (either at 12 or 16 dynes/cm<sup>2</sup>, equivalent to shear rates of 1200 and 1600 s<sup>−1</sup>, respectively). This exaggerated procoagulant activity was unrelated to inherent differences in membrane function or phospholipid content because the maximal prothrombinase activities between wild type and mIQGAP1<sup>−/−</sup> platelets were essentially identical (Fig. 3a). Similarly, there was no evidence that the enhanced procoagulant activity could be explained by inherent differences in microvesiculation as determined by flow cytometric analyses.

More exhaustive experiments were completed using platelets activated by shear stress of 16 dynes/cm<sup>2</sup> (corresponding to ~2.5 times normal shear stress in coronary arteries, or ~40% degree of stenosis). Because the prothrombinase measurement of platelet procoagulant activity reflects the composite sum generated from microvesicular and intact platelets (23), parallel determinations were made of the proportion of the total activity derived from platelet microparticles. As shown in Fig. 4, by using larger numbers of mouse platelets activated by moderate shear stress (n = 12 in each arm, with male:female ratios of 1:1), mIQGAP1<sup>−/−</sup> platelets demonstrated an enhanced ability for prothrombinase generation, differences that were highly significant compared with wild-type controls (p < 0.0001). Although microvesiculation is known to accompany high shear activation, minimal (and essentially comparable) microparticle formation was evident in both mIQGAP1<sup>−/−</sup> and wild-type platelets activated by low-shear (Fig. 5), consistent with prior observations (22). Moreover, the prothrombinase activity was primarily found in the intact platelets from both groups of mice, with an identical fraction (~26%) accounted for.
IQGAP1 Regulates Platelet Procoagulant Activity

Fig. 3. Quantitative prothrombinase determinations of mIQGAP1−/− and wild-type platelets. a, aliquots of gel-filtered platelets (4 × 10⁶/well) in HBMT were loaded into individual wells and activated by agonists (or shear) for 10 min followed by prothrombinase determinations as described under “Materials and Methods.” Results are expressed as the mean ± S.E. from duplicate wells from one complete set of representative studies (repeated once), except for shear-determinations, which are from one well. The inset shows the maximal attainable prothrombinase activity using 5 μM ionomycin (n = 10 genotype). b, flow cytometric scattergrams (lower panels) demonstrate no differences in annexin-V binding or PMP formation between wild-type (solid lines) and mIQGAP1−/− (light brown) platelets maximally stimulated using 5 μM ionomycin (representative dot plot is shown in upper right panel). The upper left panel dot plot demonstrates unstimulated platelets. PE, phycoerythrin.

by platelet microparticles. Thus, although IQGAP1 is known to have actin-polymerizing functions that could be involved in the cytoskeletal actin reorganization known to accompany microvesiculation, mIQGAP1 deficiency is uniquely associated with enhanced shear-associated platelet prothrombinase activity, without a concomitant defect in microvesiculation.

mIQGAP1−/− Procoagulant Activity Is Disjoined from \([\text{Ca}^{2+}]\), and Coupled to the Platelet Exocytic Pathway—To fur-
IQGAP1 Regulates Platelet Procoagulant Activity

What is the mechanism by which IQGAP1 modulates platelet procoagulant activity, and how is this related to the exaggerated \([\text{Ca}^{2+}]\) transients? Previous observations have demonstrated complex relationships among \(\text{Ca}^{2+}\), calmodulin, and structural domains of IQGAP1. Indeed, \(\text{Ca}^{2+}\) can directly associate with IQGAP1 (24), and \(\text{Ca}^{2+}/\text{calmodulin}\) binding modulates the functional interactions of IQGAP1 with actin and cdc42 (25, 26). Furthermore, the IQ motifs display differential binding to apocalmodulin compared with \(\text{Ca}^{2+}/\text{calmodulin}\) (27). This observation implies that IQGAP1 may facilitate \(\text{Ca}^{2+}\) association to calmodulin-bound IQGAP1, a model that may explain the exaggerated \([\text{Ca}^{2+}]\) release in IQGAP1-null platelets. Irrespective of the mechanism, this aberrant \([\text{Ca}^{2+}]\) response appears unrelated to the enhanced procoagulant phenotype evident in shear-induced IQGAP1\(^{-/-}\) platelets, data that are entirely consistent with previous observations disjoining the intracytosolic calcium release from the procoagulant capacity of the activated platelet membrane (2, 4, 20, 21). Rather, our data establish that IQGAP1 functions in a calcium-
independent secretory pathway of α-granule release, specifically activated by shear stress.

Regulated platelet exocytosis proceeds through a sequence involving granule docking and fusion, utilizing core SNARE (soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor)-related molecules that are functional in neuronal secretion and conserved in yeast (28). To date, there is no evidence that IQGAP1 functions within this complex, although the S. cerevisiae Iqg1p homologue is known to interface proteins regulating polarity-dependence with those involved in exocytosis/secretion and cytokinesis (29). Alternatively, IQGAP1 could modulate α-granule secretion by interactions with microtubules or microtubule-associated proteins (known to be important for platelet secretion (28), a prime candidate being CLIP-170 (8)). Recent evidence would suggest that an IQGAP1/CLIP-170 scaffold complexed with [GTP]-bound rac1/cdc42 functions as a link between microtubules and the cortical actin meshwork, thereby providing a calcium-independent, integrated signaling complex that could normally function to negatively regulate the early stages of granule exocytosis.

What is the mechanoreceptor linked to platelet IQGAP1 activation, and how does IQGAP1 become activated during shear stress? Although various studies have delineated key IQGAP1 structural domains mediating biochemical interactions with regulatory proteins in vitro, our data provide the first evidence for a physiological signal (i.e., shear) directly linked to IQGAP1 activation. Previous data have established a role for the von Willebrand factor/GPIbα mechanoreceptor axis in shear-induced conformational activation of αIIIβ3, although the role of this agonist-receptor complex (and its signaling pathway) in the generation and assembly of the prothrombinase complex remains incompletely delineated (30). Our shear loop did not include exogenous von Willebrand factor, suggesting that the intracellular signaling pathway occurs independently of von Willebrand factor/GPIbα ligation, although we cannot definitively exclude the possibility of GPIbα activation from endogenous platelet-derived von Willebrand factor. Interestingly, the physical properties of the phospholipid bilayer may be sufficient for shear stress-induced activation of membrane-bound G proteins in the absence of cell surface receptors (31), thereby providing a direct link to IQGAP1 via GTP-charging of rac1 and/or cdc42. Finally, although IQGAP1 specifically modulates the kinetics of prothrombinase assembly, our data also suggest that IQGAP1 deficiency results in progressive procoagulant activity over time, imputing a regulatory role for IQGAP1 in sustaining cell surface thrombin generation. These data implicate IQGAP1 in a fundamental signaling mechanism that regulates platelet procoagulant activity and presumably normal hemostasis; further characterization of

Fig. 5. Dissection of PS-exposing versus exocytic pathways of shear activation. GFP (4 × 10^4/μl) were activated by shear (16 dynes/cm²), and at various time points, samples were removed and analyzed by flow cytometry using annexin V-phosphatidylethanolamine (a) or anti-CD62P-selectin (b). a, scattergrams of annexin V-phosphatidylethanolamine labeling, or forward/side scatter distribution in nonfluorescent cells are displayed. Solid lines, wild type; light brown, mIQGAP1/−/−. For all experiments and time points, a total of 10,000 gated events were quantified. Results represent a complete and representative set of experiments, repeated on two separate occasions.
this signaling pathway should identify novel targets capable of modulating platelet procoagulant function.

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