Primary Platelet Signaling Cascades and Integrin-mediated Signaling Control ADP-ribosylation Factor (Arf) 6-GTP Levels during Platelet Activation and Aggregation*

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Previous studies showed that ADP-ribosylation factor 6 (Arf6) is important for platelet function; however, little is known about which signaling events regulate this small GTP-binding protein. Arf6-GTP was monitored in platelets stimulated with a number of agonists (TRAP, thrombin, convulxin, collagen, PMA, thapsigargin, or A23187) and all led to a time-dependent decrease of Arf6-GTP. ADP and U46619 were without effect. Using inhibitors, it was shown that the decrease of Arf6-GTP is a direct consequence of known signaling cascades. Upon stimulation via PAR receptors, Arf6-GTP loss could be blocked by treatment with U-73122, BAPTA/AM, Ro-31-8220, or Go6976, indicating requirements for phospholipase C, calcium, and protein kinase C (PKC) α/β, respectively. The Arf6-GTP decrease in convulxin-stimulated platelets showed similar requirements and was also sensitive to piceatannol, wortmannin, and LY294002, indicating additional requirements for Syk and phosphatidylinositol 3-kinase. The convulxin-induced decrease was sensitive to both PKCα/β and δ inhibitors. Outside-in signaling, potentially via integrin engagement, caused a second wave of signaling that affected Arf6. Inclusion of RGDS peptides or EGTA, during activation, led to a biphasic response; Arf6-GTP levels partially recovered upon continued incubation. A similar response was seen in β3 integrin-null platelets. These data show that Arf6-GTP decreases in response to known signaling pathways associated with PAR and GPVI. They further reveal a second, aggregation-dependent, process that dampens Arf6-GTP recovery. This study demonstrates that the nucleotide state of Arf6 in platelets is regulated during the initial phases of activation and during the later stages of aggregation.

Platelet activation is initiated through several classes of membrane receptors, which are stimulated by agonists produced at the vascular lesion (1–3). A second wave of signaling, caused by engagement of integrins, occurs as platelets bind to the lesion surface and aggregate (4). Together, these plasma membrane proteins initiate the platelet processes important for thrombosis (e.g. adhesion, spreading, secretion, and clot retraction). Small GTP-binding proteins, specifically members of the Ras superfamily, link signaling events from various platelet receptors to defined outcomes, such as shape change (5–7), aggregation (8, 9), and secretion (10–12). Rab proteins play roles in granule secretion, with Rab4 and Rab6 being involved in alpha granule release (10, 11) and Rab27a/b in dense core granule release (12, 13). RaLa is activated in response to various stimuli (14–16) and may play a role in secretion by anchoring the exocyst complex to specific membrane sites (17). Rap1 plays a role in integrin αIbβ3 activation (8, 9). Rho family GTPases (Rho, Rac, and Cdc42) play roles in platelet phosphoinositide signaling and in the regulation of the actin cytoskeleton (5–7). While these small GTP-binding proteins are clearly important to platelet function, it is equally clear that other small G proteins are present and functional in platelets (18).

The ADP-ribosylation factor (Arf) family are Ras-related, small GTPases that affect both vesicular transport and cytoskeletal dynamics (19, 20). Based on their primary sequences, this family is divided into three classes, with Arf6 as the only member of class III (19). Arf6-GTP is considered the “active state” and can interact with downstream effectors, such as phospholipase D (PLD) (21), phosphatidylinositol 4-phosphate 5-kinase type IIa (22), and arfap3 (23, 24), resulting in the recruitment of these effectors to the plasma membrane. The Arf6 GDP/GTP cycle is mediated by interactions with guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The large number of Arf-GEF and -GAP proteins have been discussed in recent reviews where it was noted that, unlike other small GTPases, Arf functions are generally not mediated solely by the GTP-bound state but through its cycling between states (19, 20, 25, 26). The effects that Arf6 has on the secretion and actin dynamics in nucleated cells make it an ideal candidate for function in platelets. Arf6 influences cortical actin and is important for spreading, ruffling, migration, and phagocytosis (reviewed in Ref. 19). Our previous work (27) showed that Arf6 is present on platelet membranes and is important for platelet function.

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2 The abbreviations used are: Arf, ADP ribosylation factor; PKC, protein kinase C; P13K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PLD, phospholipase D; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; PGILα, prostaglandin I2; DAG, diacylglycerol; HGF, hepatocyte growth factor; TRAP, thrombin receptor-activating peptide; PMA, phorbol 12-myristate 13-acetate; PAR, protease-activated receptor.
Unlike other small G proteins, the Arf6 GTP-bound form is readily detectible in resting platelets and upon activation with collagen or convulxin there is a rapid conversion to the GDP-bound form. Acetylated peptides, which mimic the myristoylated N terminus of Arfs have been used as isoform-specific inhibitors (28). In platelets, a myristoylated-Arf6 (myr-Arf6) peptide specifically blocks the activation-dependent loss of Arf6-GTP. This peptide also blocks aggregation, spreading on collagen, and activation of the rho family of GTPases. Other GTPases, such as Ras and Rap, were unaffected. The simplest explanation for these data is that platelet activation stimulates the GTPase activity of Arf6, perhaps through activation of an Arf6-GAP. Alternatively,platelet activation could affect an Arf6-GEF thus reducing the production of Arf6-GTP. Regardless of mechanism, disruption of the activation-dependent loss of Arf6-GTP, with the myr-Arf6 peptide, profoundly affects the actin-based cytoskeletal rearrangements associated with platelet activation. While our initial report (27) established a role for Arf6 in platelet function, it was not clear what platelet signaling events were required to induce the loss of Arf6-GTP.

In this article, we delineate the signaling cascades required for the activation-dependent loss of Arf6-GTP. We show that the Arf6-GTP to -GDP conversion was stimulated by primary agonists (thrombin, TRAP, collagen, or convulxin) but not by ADP or U46619. The decrease in Arf6-GTP, downstream of thrombin and convulxin, required PLC, and PKC activity. Loss of Arf6-GTP, via stimulation of GPVI with convulxin, additionally required Syk and PI3K activities. Pretreatment with passivators, nitric oxide (NO), and prostaglandin I2 (PGI2) blocked thrombin- and convulxin-induced loss of Arf6-GTP. Further experiments suggested a role for “outside-in” signaling, especially once platelet aggregates begin to form. Inclusion of RGDS peptide, EGTA, or the deletion of the β3 integrin had only minimal effects on the initial loss of Arf6-GTP but led to the partial recovery of Arf6-GTP levels. This biphasic change in Arf6-GTP levels was not seen when aggregation was allowed to occur normally. Taken together, these data show that the Arf6 nucleotide state is responsive to both initial agonist-mediated signaling and to a second wave of integrin-mediated signaling that occurs upon aggregation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Thapsigargin, BAPTA/AM, PP2, piceatannol, Ro-31-8220, G60976, and A23187 were from Calbiochem. U46619, DEA, and DEA-NONO were from Cayman Chemical (Ann Arbor, MI). Apyrase, PMA, wortmannin, genistein, and prostaglandin I2 (PGI2) were from Sigma. The β3-/- (29) and wild-type control mice were a generous gift from Dr. Susan Smyth (Dept. of Internal Medicine, University of Kentucky College of Medicine, Lexington, KY).

Washed Human Platelet Preparation—Freshly banked platelets were obtained as units from the Kentucky Blood Center (Lexington, KY). Platelet-rich plasma (PRP) was isolated in the presence of 0.37 units/ml apryrase and 10 ng/ml PGI2 by centrifugation at 150 × g for 10 min at 20 °C. PRP was centrifuged at 900 × g for 10 min, and pelleted platelets were resuspended in HEPES/Tyrode’s buffer (20 mM HEPES/NaOH, pH 6.5, 128 mM NaCl, 2.8 mM KCl, 1 mM MgCl2, 5 mM d-glucose, 12 mM NaHCO3, 0.4 mM Na2HPO4, containing 1 mM EGTA, 0.37 units/ml apryrase, and 10 ng/ml PGI2). Platelets were washed and resuspended in HEPES/Tyrode’s buffer (pH 7.4) without EGTA, apryrase, or PGI2. The final platelet counts were adjusted to 4 × 108 platelets/ml, unless otherwise indicated.

Blood Collection and Washed Mice Platelet Preparation—Mice were euthanized by CO2 inhalation. Blood was collected from the right ventricle and was mixed with sodium citrate to a final concentration of 0.38%. The citrated blood was mixed with an equal volume of PBS, pH 7.4 and incubated with PGI2 for 5 min. Platelet-rich plasma (PRP) was prepared by centrifugation at 250 × g for 10 min. The PRP was centrifuged at 500 × g for 10 min, and the platelet pellets were gently resuspended in HEPES/Tyrode’s buffer in the presence of 0.37 units/ml apryrase, 10 ng/ml PGI2, and 1 mM EGTA. Platelets were washed and finally resuspended in HEPES/Tyrode’s buffer (pH 7.4) without EGTA, apryrase, or PGI2. The final platelet counts were adjusted to 4 × 108 platelets/ml.

Platelet Aggregation—Platelet suspension (500 μl) were stirred at 37 °C in a Lumi-Dual aggregometer (Model 460Vs, Chrono-Log) for 2 min prior to addition of U46619 (1 μM), ADP (10 μM), TRAP (20 μM), thrombin (0.3 units/ml), collagen (5 μg/ml), convulxin (0.1 μg/ml), A23187 (1 μM), or PMA (200 nM). Aggregation was measured as the percent change in light transmission where 100% refers to the transmittance of platelet-poor plasma (PPP) or HEPES/Tyrode’s buffer, and aggregation curves were acquired using a Model 810 Aggro/Link computer interface and Aggro/Link software (Chrono-Log).

Platelets were generally pretreated with the indicated inhibitors at 37 °C for 2–5 min and then stimulated with agonists for the indicated times.

Arf6-GTP Pull-down Assay—Small GTPase pull-down assays were done using the Arf-GTP-specific GST-human GGA3VHS-GAT(1–313) as described (27). Briefly, reactions were stopped with 2× ice-cold HEPES lysis buffer (20 mM HEPES/NaOH, pH 7.4, 128 mM NaCl, 9 mM MgCl2, 2% Triton X-100, 0.2% SDS, 1% deoxycholic acid, 20% glycerol, 2× EDTA-free protease inhibitor mixture). The lysates were cleared by centrifugation, and the supernatants were incubated with an excess of glutathione-agarose beads bound to GST-GGA3. The bead-bound complexes were washed three times with HEPES-wash buffer (20 mM HEPES/NaOH, pH 7.4, 128 mM NaCl, 3 mM MgCl2, 1% Triton X-100, 100% glycerol). Bound Arf6-GTP was eluted, separated by 12.5% SDS-PAGE, and detected by Western blot. Vistra ECF (Amersham Biosciences, Piscataway, NJ) was used for visualization, and images were obtained using Typhoon 9400 (Amersham Biosciences). A second Western blot of a portion of the total solubilized platelet extract was...
Arf6 Activation in Platelets

FIGURE 1. Time course of Arf6-GTP decrease in agonist-stimulated platelets. Washed platelets were stimulated for increasing times with the indicated agonists: A, TRAP (20 μM, black bar), thrombin (0.3 units/ml, gray bar); B, convulxin (0.1 μg/ml, black bar), collagen (5 μg/ml, gray bar); C, Ca²⁺ ionophore, A23187 (1 μM, black bar), PMA (200 nM, gray bar); D, ADP (10 μM, black bar), U46619 (1 μM, gray bar). After lysis and centrifugation, the supernatants were assayed for Arf6-GTP. Arf6-GTP and total Arf6 levels were detected by quantitative Western blotting, quantified as described under “Experimental Procedures,” and used to generate a ratio of Arf6GTP/Arf6_total for each time point. The ratio at time 0 was set to 100% and used to normalize the data. The Western blots shown are for the Arf6-GTP.

RESULTS

Activation-dependent Loss of Arf6-GTP in Platelets—In Choi et al. (27), we observed that Arf6-GTP levels were higher in resting platelets and decreased upon stimulation. To extend these studies, we determined the time course of this decrease in response to both primary and secondary platelet agonists. Stronger, primary agonists all stimulated the loss of Arf6-GTP. PAR-directed activation (TRAP or thrombin), showed a rapid decrease in Arf6-GTP reaching a maximum by 30 s. No recovery of Arf6-GTP was seen in reactions incubated for 5 min (Fig. 1A). As described before, GPVI-directed agonists (convulxin or collagen), also induced a decrease in Arf6-GTP; though the response to collagen was somewhat slower (Fig. 1B). The Ca²⁺ ionophore, A23187, and the PKC activator, PMA, both led to a decrease in Arf6-GTP in a comparable time frame (Fig. 1C). A23187 is a strong activator of platelet aggregation and secretion. PMA is not (induced <30% aggregation); however, it directly activates platelet PKC isoforms (28). Taken together, these data show that PAR- and GPVI-mediated signaling cascades cause a decrease in Arf6-GTP levels.

Previous studies showed that secondary agonists, ADP and U46619 (thromboxane A2 analogue), were individually unable to reduce Arf6-GTP levels. To confirm these data, platelets were stimulated with 10 μM ADP or 1 μM U46619 (which induced <40% aggregation). No significant effect on Arf6-GTP levels was seen at any of the time points tested (Fig. 1D).

Platelet passivators dampen platelet activation through the production of cyclic nucleotide second messengers, which activate specific kinases and modulate platelet calcium levels (30, 31). PGI₂ and NO are both considered passivators because of their effects on cyclic AMP and cyclic GMP levels, respectively. Neither compound had any effect on Arf6-GTP levels in resting platelets (Fig. 2A). However, when platelets were pretreated for 2 min with the passivators, either alone or in combination, there was an inhibition of Arf6-GTP loss in response to both convulxin and thrombin (Fig. 2, B and C, respectively). These data show that passivator pretreatments affect the activation-dependent loss of Arf6-GTP, but have no effect on its levels in resting cells.

Signaling Steps Required for Activation-dependent Loss of Arf6-GTP—Given the data above, we sought to determine what elements of the known platelet signaling cascades were required for the activation-dependent loss of Arf6-GTP. For this analysis, we used inhibitors of the kinases and phospholipases that have been reported to be involved in the cascades downstream of PAR- and GPVI-mediated activation. In all cases, the effect of the inhibitors was monitored by aggregometry to assure efficacy.

Syk is a non-receptor tyrosine kinase that is activated just downstream of GPVI (32). The Syk inhibitor piceatannol (10 μM) blocked convulxin-induced loss of Arf6-GTP but had no
effect on thrombin-induced Arf6-GTPase activation (Fig. 3A). Consistently, piceatannol blocked convulxin-induced aggregation but not that induced by thrombin (data not shown). PI3K activity has also been shown to be an important element of the signaling cascades proximal to GPVI but not PAR receptors (33). Two different PI3K inhibitors, wortmannin and LY294002, blocked the loss of Arf6-GTP in response to convulxin stimulation, but not in response to thrombin (Fig. 3B). This differential effect was mimicked in the aggregation traces (data not shown). These data show that Arf6 is part of the expected signaling cascades initiated by GPVI and is downstream of both Syk and PI3K activation.

Activation of PLC isoforms and thus the production of IP3 and DAG are critical for platelet activation (2). Given the data above, the next step was to determine if PLC activity was required for decrease of Arf6-GTP. Platelets were treated with increasing concentrations of the PLC inhibitor U-73122 or its inactive isomer U-73343 and then stimulated with thrombin for 5 min (Fig. 4A). U-73122 inhibited loss of Arf6-GTP in a dose-dependent manner while the inactive isomer had no effect. U-73122, at 10 μM, also blocked the convulxin-induced loss of Arf6-GTP (Fig. 4B). These results suggest that the activation-dependent loss of Arf6-GTP is downstream of PLC.

Conversely, thapsigargin treatment (1 μM for 5 min), which causes an unregulated release of calcium stores, led to a decrease of Arf6-GTP (Fig. 4, C and 4D). These data demonstrate the importance of intra-platelet calcium to the activation-dependent loss of Arf6-GTP and are consistent with the effect of A23187 seen in Fig. 1C.

Given the data above, we next focused on the role of PKC. Initially, we tested the effect of the broad specificity, PKC inhibitor, Ro-31-8220. In both thrombin- and convulxin-stimulated platelets, Ro-31-8220 (1 μM) treatment blocked Arf6-GTP loss (Fig. 4, C and D). Ro-31-8220 also blocked the effect of thapsigargin (Fig. 4D) underlining the interplay between calcium and PKC activation. Further experiments used more isoform-specific PKC inhibitors: Go6976 for λ/δ isoforms (35) and rottlerin for PKCζ (35). In Fig. 5, Go6976 (1 μM) blocked thrombin- and convulxin-induced loss of Arf6-GTP as did Ro-31-8220. Interestingly, rottlerin (10 μM) only partially affected the Arf6-GTP loss in
response to thrombin (62.5% decrease) but completely blocked the response to convulxin. These data suggest that PKC\(\beta\) is only partially required for the thrombin-induced decrease of Arf6-GTP but is essential for convulxin-induced loss of Arf6-GTP.

In summary, these data show that the loss of Arf6-GTP in response to platelet activation is dependent on PLC, intra-platelet calcium, and PKC. Additionally, Syk and PI3K are required for loss of Arf6-GTP levels in response to GPVI-directed agonists. These data show that the activation-dependent loss of Arf6-GTP is responsive to known elements of the platelet signaling pathways and suggests that Arf6 is a bona fide part of these cascades.

Role of Src Family Kinases in Activation-dependent Loss of Arf6-GTP—Other non-receptor tyrosine kinases (i.e. Src-family kinases) function downstream of both GPVI- and PAR-activated signaling cascades (36) as well as downstream of “outside-in” integrin-initiated signaling (37). One general tyrosine kinase inhibitor (genistein) and one Src-family kinase inhibitor (PP2) were tested for their effects on the activation-dependent decrease of Arf6-GTP. Both inhibitors affected the loss of Arf6-GTP in response to either thrombin or convulxin (5-min post-stimulation; Fig. 6A), though the effects were less robust than what has been seen with other inhibitors in this study. To further probe the role of Src-family kinases, platelets were incubated with PP2 (10 \(\mu\)M), then activated with either convulxin or TRAP for increasing times under stirring conditions. PP2 affected the loss of Arf6-GTP in response to both agonists (Fig. 6, B and C). The effect on convulxin-stimulated Arf6-GTP loss (Fig. 6B) was more robust, consistent with the proposed role of Src-family kinases immediately downstream of GPVI. The effect on TRAP-stimulated Arf6-GTP loss had a biphasic time course. Initial loss (\(t < 30\) s) of Arf6-GTP was not inhibited as robustly as was seen at the latter stages (Fig. 6C). This suggested that Src-family kinases were not essential for the first phases of PAR activation-initiated Arf6-GTP loss (\(t < 30\) s) but could play a role as platelets continue to form aggregates (\(t > 60\) s). This is the first indication that Arf6-GTP levels might be responsive to two waves of platelet signaling, one initiated by agonists and one during aggregation.

Potential Role of Integrins in Controlling Arf6-GTP Levels—Given the effect of PP2 on Arf6-GTP levels in response to TRAP, we examined the effect of platelet aggregation on Arf6-GTP levels. Platelet aggregation is driven, in large part, by the
inside-out" activation of the integrin αIIbβ3, which increases its affinity for fibrinogen. Once fibrinogen is bound, "outside-in" signaling through the integrin occurs to promote further aggregation and clot retraction. Fibrinogen binding can be disrupted by chelating calcium or by inclusion of a competing peptide. Both strategies were used to test the effect of platelet aggregation on loss of Arf6-GTP. Upon stimulation with TRAP, inclusion of EGTA (Fig. 7A) slightly affected the initial phase of aggregation and clot retraction.
the response (t < 30 s) but led to a recovery of Arf6-GTP at the later stages of the time course. This biphasic response in Arf6-GTP levels mirrors that seen with TRAP-stimulated platelets treated with PP2 (Fig. 6C). Addition of RGDS peptide (1 mM), to block integrin engagement, led to a similar biphasic response when either thrombin (Fig. 7B) or convulxin (Fig. 7C) were used as agonists. The RGDS peptide had little effect on the initial stages of the Arf6-GTP decrease but led to a recovery of Arf6-GTP upon longer incubation (t > 30 s). In all treatment groups (+EGTA or +RGDS peptide), platelets are activated, as demonstrated by their shape change in aggregometry traces, but fail to aggregate indicating the efficacy of the treatment (data not shown).

Upon platelet aggregation, a number of signaling events occur through engagement of surface receptors. Activated integrins can mediate “outside-in” signaling to promote contact-dependent processes (38). Ephrins can also initiate platelet signaling cascades through platelet-platelet contacts (39). Our previous work (27) and data shown here suggest that integrin engagement is not required for the initial (t < 30 s), agonist-stimulated loss of Arf6-GTP, but may play a role in subsequent steps that further reduce Arf6-GTP or suppress its recovery. To directly address the role of integrin signaling, we examined the time course of Arf6-GTP loss in mouse platelets deficient in β3 (29) (Fig. 8). Western blotting of platelet extracts from these mice confirmed the loss of the β3 subunit (Fig. 8B, inset). In aggregometry traces the β3−/− platelets showed shape change but no aggregation occurred in response to 0.3 units/ml thrombin (Fig. 8B). Wild-type and β3−/− platelets were examined for agonist-dependent loss of Arf6-GTP in response to thrombin. In Fig. 8A, wild-type mouse platelets showed a time-dependent loss of Arf6-GTP similar to human platelets. Once decreased, Arf6-GTP levels did not recover during the experimental time course. Loss of Arf6-GTP in the β3−/− platelets was unaffected during the initial phase of activation (t < 60 s), but Arf6-GTP levels recovered, almost to resting levels, upon prolonged incubation. This implies that β3 is important for the prolonged depression of Arf6-GTP levels seen in stimulated platelets.

**DISCUSSION**

In this article, we delineate some of the signaling steps required for the activation-dependent decrease of Arf6-GTP levels in platelets. We show that primary agonists directed through G-protein-coupled PAR receptors or through tyrosine kinase-coupled GPVI induced a decrease in Arf6-GTP. The PAR-initiated loss of Arf6-GTP required PLC, Ca2+, and PKC. GPVI-stimulated loss of Arf6-GTP also required PI3K, Syk, and Src family kinases. Such data show that Arf6-GTP levels are responsive to the known signaling cascades present and active in stimulated platelets. While this is perhaps not surprising, the data are significant because they are the first demonstration that Arf6 is a responsive element of the platelet signaling machinery and they position Arf6 downstream of PKC.

The initial surprise from our findings is that Arf6 is affected by a second, aggregation-dependent, signaling process that dampens recovery of Arf6-GTP levels. When aggregation was disrupted by a calcium chelator, a RGDS peptide, or deletion of β3 integrins, the Arf6 nucleotide state showed a biphasic response. The initial conversion to Arf6-GDP was unaffected; but, there was a recovery of the GTP-bound state upon continued incubation. The implications from these observations are that the Arf6 nucleotide state is not only responsive to the initial agonist-dependent signaling but is also affected by a second signaling cascade initiated by the platelet-platelet or platelet-matrix contacts occurring during aggregation. This second signaling event prevents Arf6-GTP levels from returning to that seen in resting platelets. While engagement of integrins and integrin-mediated outside-in signaling is a likely mechanism mediating this second wave, we cannot formally discard a role for other signaling pathways such as ephrins or cadherins. Further experiments will be required to define which set of platelet contacts are directly affecting the Arf6 nucleotide state.

The GTP/GDP-bound state of Arf6 is controlled by a range of exchange factors (GEFs) and GTPase activators (GAPs). Not only do these proteins control the Arf6 nucleotide state, but often, they are themselves effectors controlled by Arf6. At present, little is known about which are present in platelets. ASAP1, a GAP, was purified from platelet extracts (40) and Western blotting has shown that ACAP-1 and -2 are also present.3 One role for these GAPs is thought to be in the recycling plasma membrane proteins (41). The presence of GIT1 has been reported (42) and confirmed in our laboratory by Western blotting.3 GIT1, through its interactions with PIX and paxillin, may affect the actin cytoskeleton via control of Cdc42 and Rac (43, 44). Even less is known about which Arf-GEFs are present, though ARNO was detected by Western blotting.3 This lack of knowledge limits any speculation on the mechanisms of how Arf6 nucleotide state is controlled in platelets. As stated earlier, the simplest explanation for the acute decrease in Arf6-GTP is via the activation of a GAP. Studies with the inhibitory myr-Arf6 peptide are consistent because it can inhibit interactions between Arf6 and its GAPs (45), and it does block Arf6-GTP loss in activated platelets (27). However, there are not sufficient data to unequivocally define such a model. The second, aggregation-initiated effect on Arf6 could

3 W. Choi, unpublished data.
Arf6 Activation in Platelets

occur via activation of a GAP or inactivation of a GEF. Perhaps informative here is the report that GIT1 is tyrosine-phosphorylated in activated platelets and that such phosphorylation is blocked by treatment with RGDS peptide (42). A detailed catalogue of Arf6 effectors in platelets will be required before more definitive mechanisms can be proposed.

The activation-dependent decrease in Arf6-GTP is puzzling given what is known of other small-GTP-binding proteins in platelets. We and others have shown that Rho, Rac, Cdc42, Ral, and Rap, are all “activated” to their GTP-bound states upon platelet stimulation. Arf6 is the opposite; its GTP-bound state is found in resting platelets and is lost upon activation. This is not the result of sequestration of Arf6-GTP from our pull-down assay because most (>90%) of the Arf6 is solubilized by our assay conditions (see “Experimental Procedures”). A clue to this unique behavior may lie in the role(s) of Arf6 in nucleated cells. Arf6 affects the cycling of membrane proteins (e.g. integrins and cadherins) between an endosomal compartment and the plasma membrane (46–48) and thus could control cell-cell or cell-matrix contacts. MDCK epithelial cells are a demonstrative example of this. MDCK monolayers have well-defined cell-cell contacts and low Arf6-GTP levels. Upon stimulation with hepatocyte growth factor (HGF), Arf6-GTP levels increase, the adherens junctions dissipate, and the cells begin to migrate (49, 50). Expression of the Arf6-GTPase mutant (Q67L) promotes adherens junction disassembly and cell migration, implying that Arf6-GTP levels are important for dispersing the monolayer and allowing cells to respond as individuals. Conversely, expression of the dominant-negative Arf6 (T27N) blocks cell migration as well as HGF-induced internalization of cadherins. Thus a shift in the steady-state to Arf6-GDP promotes or allows cell-cell contacts; a shift to Arf6-GTP promotes dissolution of cell-cell contacts and cell migration. Using these observations as a guide, we posit that Arf6-GTP/GDP cycling in platelets is a guide, we posit that Arf6-GTP/GDP cycling in platelets may be part of the process that maintains resting, un-aggregated platelets. When the steady state is shifted toward Arf6-GTP, the formation of platelet-platelet and platelet matrix contacts are limited. This could be through effects on the trafficking of platelet surface proteins (e.g. integrins or cadherins which are present in platelets; Ref. 51). Upon stimulation, the Arf6 steady state shifts to the GDP-bound form, which allows/promotes formation of stable platelet-platelet and platelet-matrix contacts. Such an effect would be an important aspect of clot stabilization.

While these concepts are clearly speculation, our hypothesis suggests future experiments to probe the role of Arf6 in aggregation and clot retraction. One might predict that drugs which affect Arf6 GTP/GDP cycling would shift the steady state and thus affect platelet function. Preliminary studies treating platelets with an Arf6-GEF inhibitor, specific for cytosolins (52), show decreased Arf6-GTP and a prothrombotic effect. Conversely, Arf6-GAP inhibitors are expected to block aggregation. Indeed the myr-Arf6 peptide, which mimics the acylated N terminus and blocks activation-dependent loss of Arf6-GTP, does block aggregation (27) and clot retraction. Further studies and refinement of Arf6-directed inhibitors will be required to definitively address the details of this model.

This work expands our understanding of a new element of the platelet activation process, the Ras-like GTPase, Arf6. Our previous work (27) showed the importance of Arf6 in platelet function. The present studies show that Arf6 is dynamically regulated during at least two stages of thrombosis. There is an initial decrease in Arf6-GTP initiated by known elements of agonist-driven platelet activation pathways. Additionally, the Arf6 nucleotide state is affected by a second process occurring during platelet aggregation. This, in broad outline, establishes at least two of the signaling events upstream of Arf6. Future experiments will now need to focus on which Arf6-GEFs and -GAPs are present in platelets and how they are affected by the signaling pathways described here. Other experiments will be needed to identify the Arf6 effectors present in platelets and to determine how these effectors, under Arf6 control, contribute to platelet function.

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Arf6 Activation in Platelets

MAY 2, 2008 • VOLUME 283 • NUMBER 18 • JOURNAL OF BIOLOGICAL CHEMISTRY

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