Activation of Rap1B by G\textsubscript{i} Family Members in Platelets*

Donna Woulfe‡, Hong Jiang‡, Richard Mortensen‡, Jing Yang‡‡, and Lawrence F. Brass‡¶

From the ‡Departments of Medicine and Pharmacology and the Center for Experimental Therapeutics, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and §Department of Physiology and Medicine (Endocrine), University of Michigan, Ann Arbor, Michigan 48109

It has become increasingly appreciated that receptors coupled to Go\textsubscript{i} family members can stimulate platelet aggregation, but the mechanism for this has remained unclear. One possible mediator is the small GTPase, Rap1, which has been shown to contribute to integrin activation in several cell lines and to be activated by a calcium-dependent mechanism in platelets. Here, we demonstrate that Rap1 is also activated by Go\textsubscript{i} family members in platelets. First, we show that platelets from mice lacking the Go\textsubscript{i} family member Go\textsubscript{i3} (which couples to the \(\alpha_{2A}\) adrenergic receptor) are deficient in epinephrine-stimulated Rap1 activation. We also show that platelets from mice lacking Go\textsubscript{i} (which couples to the ADP receptor, P2Y12, exhibit reduced Rap1 activation in response to ADP. In contrast, platelets from mice that lack Go\textsubscript{i2} show no decrease in the ability to activate Rap1 in response to epinephrine but show a partial reduction in ADP-stimulated Rap1 activation. This result, combined with studies of human platelets treated with ADP receptor-selective inhibitors, indicates that ADP-stimulated Rap1 activation in human platelets is dependent on both the Go\textsubscript{i}-coupled P2Y12 receptor and the Go\textsubscript{i}-coupled P2Y1 receptor. Go\textsubscript{i2}-dependent activation of Rap1 in platelets does not appear to be mediated by enhanced intracellular calcium release because no increase in intracellular calcium concentration was detected in response to epinephrine and because the calcium response to ADP was not diminished in platelets from the Go\textsubscript{i2}–/– mouse. Finally, using human platelets treated with selective inhibitors of phosphatidylinositol 3-kinase (PI3K) and mouse platelets selectively lacking the \(G\beta\gamma\)–activated form of his enzyme (PI3K\(\gamma\)), we show that G\textsubscript{i}-mediated Rap1 activation is PI3K-dependent. In summary, activation of Rap1 can be stimulated by Go\textsubscript{i} and PI3K-dependent mechanisms in platelets and by Go\textsubscript{i} and Ca\textsuperscript{2+}-dependent mechanisms, both of which may play a role in promoting platelet activation.

Formation of the platelet plug at sites of vascular injury begins with the exposure of collagen within the vessel wall. Circulating platelets adhere to collagen with the help of von Willebrand factor and become activated and release soluble mediators, such as ADP and thromboxane A\textsubscript{2}. These, in turn, along with locally generated thrombin, help to extend platelet plug formation (1). With the exception of collagen, most platelet agonists, including thrombin, ADP, thromboxane A\textsubscript{2}, and epinephrine, activate platelets by binding to one or more classes of G protein-coupled receptors on the platelet surface (2), leading to the exposure of fibrinogen binding sites on \(\alpha_{IIb}\beta_{3}\) that are required for platelet aggregation. It has been established previously that a rise in the cytosolic Ca\textsuperscript{2+} concentration is sufficient to activate \(\alpha_{IIb}\beta_{3}\). It has also been shown that most platelet agonists trigger Ca\textsuperscript{2+} entry into the cytosol by binding to receptors coupled to the heterotrimeric G protein Go\textsubscript{i}, the activated \(\alpha_{i}\) subunit of which (Go\textsubscript{i\(\alpha\)}) can activate phospholipase C\(\beta\) (3). Not surprisingly, mouse platelets lacking Go\textsubscript{i} and human platelets with a decrease in expression of this \(\alpha\) subunit have a defect in platelet function (3, 4).

In addition to Go\textsubscript{i}, human platelets express four members of the Gi family of G proteins, Go\textsubscript{i1}, Go\textsubscript{i2}, Go\textsubscript{i3}, and Go\textsubscript{z} (5–7). It has become increasingly appreciated that receptors coupled to Gi family members, like those coupled to Go\textsubscript{i}, play an important role in stimulating platelet activation, but the details of that role are less well understood (8–10). The coupling of Gi family members to the receptors for platelet agonists is selective. This is best illustrated by recent studies on platelets from genetically engineered mice lacking the \(\alpha\) subunit for a particular Gi family member. For example, of the two most highly expressed Gi proteins in platelets, Gi\textsubscript{c} couples preferentially to the \(\alpha_{2A}\) adrenergic receptor for epinephrine (11), whereas Gi\textsubscript{z} is preferred by the ADP receptor P2Y12 (12). Loss of Go\textsubscript{i\(\alpha\)} impairs platelet responses to ADP, and loss of Go\textsubscript{z} impairs the ability of epinephrine to potentiate platelet aggregation by other agonists.

Given the defect in platelet function caused by the loss of individual Gi family members, it is reasonable to ask what signaling pathways are modulated by the activation of receptors coupled to these proteins. One generally accepted role is the inhibition of cAMP formation by adenylyl cyclase because it is clear that large increases in the cAMP concentration will inhibit platelet responses to agonists. However, whereas both Go\textsubscript{i} and Go\textsubscript{z} can clearly inhibit adenylyl cyclase, this cannot be their only role because the addition of membrane-permeable adenylyl cyclase inhibitors does not cause platelet activation equivalent to that caused by stimulation of the \(\alpha_{2A}\) or P2Y12 receptors (13), nor do these inhibitors reverse the phenotype produced by loss of Go\textsubscript{i\(\alpha\)}. Therefore, one goal of this study was to identify effectors other than adenylyl cyclase downstream of Gi activation that might promote platelet activation.

Recently, the small GTPase Rap1 has emerged as a candidate regulator of integrin activation. Several investigators have already revealed a role for Rap1 in controlling activation of

1. J. Yang, J. Wu, H. Jiang, R. Mortensen, S. Austin, D. R. Manning, D. Woulfe, and L. F. Brass, manuscript in preparation.
of β2 integrins in a number of cell lines (14–16). Furthermore, it has recently been shown that constitutively active forms of Rap1A expressed as a transgene in T cells increase adhesion to fibronectin through avidity modulation of β1 and β2 integrins (17). Like other Ras family members, Rap1 is activated by the binding of GTP and inactivated by the hydrolysis of GTP to GDP. Of the two isoforms of Rap1 found in humans (Rap1A and Rap1B, which differ by only 9 amino acids), Rap1B is highly expressed in human platelets and is rapidly activated by agonists that increase the cytosolic Ca2+ concentration, provoking speculation that this small GTPase may play a role in activation of the platelet integrin αIIbβ3 (18). Indeed, a preliminary report by Bertoni et al. (19) in which dominant negative forms of Rap1 were expressed in megakaryocytes suggests that this is the case. The precise mechanism by which a rise in cytosolic Ca2+ leads to platelet integrin activation remains unclear, as does the means by which Gi family members potentiate platelet activation. The possibility that activation of Rap1 might provide a common mechanism for both Gi- and Gq-mediated platelet activation led us to investigate whether Gq family members were capable of activating Rap1 independently of Gq-stimulated calcium release.

We show here that both epinephrine- and ADP-stimulated Rap1 activation are diminished in platelets deficient in selected Gq family members and retained in platelets from mice lacking Goαi, providing evidence that Rap1 is activated by Gq family members in mouse platelets. Using ADP receptor-selective inhibitors, we further demonstrate that ADP-dependent Rap1 activation is also stimulated by Gq-coupled receptors in human platelets. The activation of Rap1 is not as robust as that seen after the addition of thrombin, but in neither case is the Gq-mediated activation of Rap1 dependent on a detectable increase in Ca2+. Finally, using human platelets treated with selective inhibitors of PI3K and mouse platelets selectively lacking the Gβγ-activated form of this enzyme (PI3Kγ), we show that Gq-mediated Rap1 activation is PI3K-dependent. These results support and extend those reported very recently by Lovat et al. (20) and complement observations on Rap1 activation by collagen by Larson et al. (21). Taken together, they demonstrate a previously unappreciated mechanism for regulation of Rap1 in platelets through a Gq-mediated pathway involving one or more isoforms of PI3K.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma unless otherwise stated. Fura-2-AM and BAPTA-AM were purchased from Molecular Probes (Eugene, OR). Monoclonal anti-Rap1 antibody was from Transduction Laboratories. LY294002 was from Biomol Research Laboratories (Eugene, OR). Monoclonal anti-Rap1 antibody was from Transduction Laboratories. GST, glutathione S-transferase; PAR, protease-activated receptor.

Results

To understand more about the mechanisms by which Gi family members contribute to platelet reactivity and to determine if potentially calcium-independent mechanisms of Rap1 activation exist in platelets, we examined Rap1 activation in human platelets activated with epinephrine, which does not elicit a detectable increase in cytosolic Ca2+. In Fig. 1, platelets were incubated with an agonist or buffer alone and lysed. Both agonists caused an increase in Rap1 activation. Thrombin was consistently the most robust stimulus (Figs. 1–4), followed by ADP and epinephrine. In human platelets, epinephrine stimulated an average 4.5-fold increase in GTP binding to Rap1 relative to buffer alone (n = 10), ADP stimulated an average 10.5-fold increase (n = 9), and thrombin stimulated a 48-fold increase (n = 6) in Rap1-GTP binding. A representative experiment is shown in Fig. 1, B and C. Fig. 1B shows the amount of Rap1-GTP precipitated with GST-conjugated Rap-GDS-RBD by each agonist relative to total Rap1 contained in 25 μl of each lysate. By immunoblotting Rap-GTP-depleted lysate from one such experiment, we calculated that ADP caused GTP loading of about 30% of total platelet Rap1, whereas thrombin stimulated about 85% of total platelet Rap1. Epinephrine, which activates α2A adrenergic receptors coupled to Gi in platelets, activated about 10% of platelet Rap1. Thrombin and ADP cause a readily detectable increase in cytosolic Ca2+. However, neither we nor earlier investigators have been able to detect a calcium transient in response to epinephrine using calcium-binding fluorescent probes such as Fura-2 (20, 25) (Fig. 2).

Dependence of Rap1 Activation—To determine if this signal was indeed dependent on a Gi family member, the ability of epinephrine to activate Rap1 was compared in Goαi/− mice and their wild-type littermates (Fig. 2A). It has previously been shown that the α2A adrenergic receptor on platelets couples to Gi and that the ability of epinephrine to potentiate aggregation in response to other agonists is greatly diminished in mice lacking Goαi (11). Whereas the wild-type mice exhibit a Rap1 activation profile similar to that seen in humans, the Goαi/− mice activate Rap normally in response to ADP and thrombin but have a diminished ability to activate Rap1 in response to epinephrine. This Gi-dependent Rap activation does not appear
intracellular calcium in response to these agonists was tested to determine whether reduced calcium mobilization could account for the diminished Rap1 response to ADP and thrombin in \( \text{G}_{\alpha_q}^{-/-} \) mice. However, calcium transients in response to 10 \( \mu \)M ADP or 1 unit/ml thrombin (the same concentrations applied in the Rap1 activation assays) were not reduced in platelets from \( \text{G}_{\alpha_q}^{-/-} \) mice compared with their wild-type littermates, making this unlikely (Fig. 3B).

To confirm that the observed \( \text{G}_q \)-dependent signals were not dependent on activation of \( \text{G}_q \), for example due to thromboxane \( \text{A}_2 \) release from treated platelets, we tested Rap1 activation in platelets from \( \text{G}_{\alpha_q}^{-/-} \) mice. Compared with their wild-type littermates, \( \text{G}_{\alpha_q}^{-/-} \) mice responded normally to epinephrine but had a slightly reduced response to ADP and a much diminished activation by thrombin (Fig. 4). It has been shown that the increase in cytosolic calcium initiated by both thrombin and ADP is entirely dependent on \( \text{G}_{\alpha_q} \) (3), an observation that we confirmed (Fig. 4B; data not shown). Therefore, the activation of Rap1 by ADP that is retained in \( \text{G}_{\alpha_q}^{-/-} \) mice is due to intracellular calcium release. These results are consistent with the idea that ADP activates two receptors on platelets, a \( \text{G}_q \)-coupled receptor (P2Y1) and a \( \text{G}_i \)-coupled receptor (P2Y12), each of which is capable of independently leading to Rap1 activation. Thrombin activates mouse platelets via PAR4 with an assist from PAR3 (26). Murine PAR4 does not appear to couple to \( \text{G}_q \) family members, and therefore the residual Rap1 activation observed by thrombin in \( \text{G}_{\alpha_q}^{-/-} \) platelets is probably due to activation of \( \text{G}_{\alpha_2} \) by released ADP. This would also account for the reduced activation of Rap1 by thrombin in \( \text{G}_{\alpha_q}^{-/-} \) mouse platelets (Fig. 3).

\( \text{G}_q \)-dependent Rap1 Activation in Human Platelets—As described above, ADP activates platelets by binding to two receptors, P2Y1 and P2Y12. To determine whether Rap1 can be activated in a \( \text{G}_q \)-dependent manner in human as well as mouse platelets, human platelets were stimulated with ADP in the presence of saturating concentrations of the P2Y1 receptor agonist A3P5PS to allow selective activation of the \( \text{G}_i \)-coupled P2Y12 receptor. Saturation of P2Y1 sites was confirmed by demonstrating complete blockade of the calcium transient initiated by the concentration of ADP used in the Rap1 activation assay (Fig. 5C). The Rap1 activation signal was diminished but retained under these conditions in both human and mouse platelets (Fig. 5, B and A, respectively). In contrast, the P2Y12-selective inhibitors MeSAMP (see Fig. 5, A and B) and ARL-66096 (data not shown) nearly abolished the activation of Rap1 by ADP, consistent with a \( \text{G}_i \)-dependent mechanism. Therefore, it appears that in both mouse and human platelets, ADP activates Rap1 predominantly via \( \text{G}_{\alpha_2} \), even though its receptors also couple to \( \text{G}_q \).

\( \text{G}_i \)-mediated Rap1 Activation Is Dependent on P13K—To begin to dissect the mechanism by which \( \text{G}_i \) family members lead to Rap1 activation, platelets stimulated with epinephrine or with ADP plus the P2Y1 antagonist A3P5PS (to restrict the ADP response to P2Y12) were exposed to inhibitors of adenylyl cyclase or P13K and tested for Rap1 activation. To determine whether \( \text{G}_i \)-dependent Rap1 activation is simply a consequence of reduced levels of cAMP in the platelets, we tested Rap1 activation in the presence of the adenylyl cyclase inhibitor SQ22536. SQ22536 at 300 \( \mu \)M inhibited cAMP production stimulated by PGI2 to levels that were comparable to the inhibition of adenylyl cyclase caused by ADP (data not shown) but failed to cause Rap1 activation or to potentiate the activation caused by epinephrine (Fig. 6A). This suggests that the ability to stimulate Rap1 is not simply a function of depressing basal cAMP concentrations.

Hirsch et al. (27) have shown that ADP-stimulated platelets...
**FIG. 2. Rap1 activation in Gαz−/− mice.** A, platelets were isolated from Gαz−/− mice or their wild-type littermates and then stimulated for 5 min at room temperature with buffer control, 10 μM epinephrine, 10 μM ADP, or 1 unit/ml thrombin. Platelets were lysed, and Rap-GTP was isolated as described under “Experimental Procedures.” Rap-GTP is shown in the top panel, and total Rap1 from 25 μl of lysate is shown in the bottom panel. This experiment was repeated five times with similar results.

B, platelets isolated from Gαz−/− mice or their wild-type littermates were loaded with 5 μM Fura-2AM to detect changes in cytosolic Ca2+ and then stimulated with 10 μM ADP followed by 10 μM epinephrine in the top panels or stimulated with 10 μM epinephrine followed by 10 μM ADP in the bottom panels. The traces shown are representative of three experiments performed with similar results.
from PI3K-γ-null mice exhibit reduced fibrinogen binding compared with wild-type platelets. Because βγ subunits released from G, family members have been shown to stimulate PI3K, we tested the effect of two PI3K inhibitors on Rap1 activation in human platelets. Again, ADP was used in combination with A3P5PS to restrict receptor activation to P2Y12. Both PI3K inhibitors wortmannin (data not shown) and LY294002 (Fig. 6) inhibited Rap1 activated by either epinephrine or ADP in the presence of A3P5PS to near background levels, indicating that one of the PI3K isoforms is likely to be involved in Rap1 activation. Platelets express more than one form of PI3K. In the experiments summarized in Fig. 7, Rap1 activation was compared in platelets from PI3K-γ−/− mice and wild-type mice of the same strain. On average, Rap1 activation induced by epinephrine, ADP, or thrombin was decreased in the PI3K-γ−/− platelets relative to those of wild-type mice, but due to variability in the results, this difference reached significance only with ADP (using a two-tailed paired t test, p ≤ 0.05; n = 5). Therefore, it is likely that more than one PI3K isoform is involved in G,-mediated Rap1 activation.

DISCUSSION

It has been clear for some time that agonists that stimulate calcium release within platelets are capable of activating the integrin αIβ3 on the platelet surface to bind soluble fibrinogen and cause platelet aggregation. Soluble agonists such as thrombin, ADP, and thromboxane A2 all stimulate release of calcium from intracellular stores by binding to receptors coupled to the heterotrimeric G protein Gq, the α subunit of which (Gq) activates phospholipase C (see Fig. 8). In fact, aggregation in response to all of these agonists is nearly abrogated in platelets from Gq-deficient mice (3). However, recently it has become increasingly apparent that activation of Gq may not be entirely sufficient to stimulating platelet activation by some agonists (13). This has been nicely illustrated in the case of ADP, which binds to two receptors on platelets: the Gq-coupled...
receptor P2Y1 and the G\textsubscript{12} coupled receptor P2Y12 (9). Mice deficient in either of these receptors fail to aggregate in response to low or moderate concentrations of ADP, indicating that stimulation of both G\textsubscript{q} and G\textsubscript{i} coupled pathways is required for full activation (8, 28, 29). Furthermore, it has recently been reported that platelets from mice lacking the G\textsubscript{12} coupled receptor, P2Y1, are still capable of binding soluble fibrinogen and aggregating through activation of P2Y12 alone (10). Finally, aggregation of platelets induced by thromboxane A\textsubscript{2} or low concentrations of thrombin is dependent upon stimulation of a G\textsubscript{i} family member by ADP released from platelet dense granules (12, 30). Thus, it appears that activation of G\textsubscript{i} coupled receptors provides an independent signal that acts synergistically with G\textsubscript{q} mediated pathways to achieve full activation of platelets.

In this study, we sought to understand the mechanisms by which signaling through G\textsubscript{i} family members mediates platelet activation. There are four known G\textsubscript{i} family members in platelets: G\textsubscript{i2}, G\textsubscript{i3}, G\textsubscript{i4}, and G\textsubscript{i1} (in relative order of expression level). At least two of these G\textsubscript{i} family members have a unique affinity for cell surface receptors: G\textsubscript{i3} preferentially couples to the \alpha\textsubscript{2A} adrenergic receptor for epinephrine (11), whereas G\textsubscript{i2} couples preferentially to the ADP receptor P2Y12 (12). The "traditional" role of all G\textsubscript{i} family members is to inhibit the enzyme adenyl cyclase, reducing the concentration of cAMP in the cell. It has been well established that high concentrations of cAMP inhibit platelet aggregation, due in part to an inhibitory effect on intracellular calcium release. However, a decrease in cAMP alone (and the resulting release of tonic inhibition on platelet activation) appears insufficient to account for all of the roles played by G\textsubscript{i} family members in the support of platelet aggregation. In particular, the ability of epinephrine to stimulate aggregation of human platelets in the absence of a detectable calcium transient (and a similar effect of stimulating the ADP receptor P2Y12 alone) suggests that there are additional functions of G\textsubscript{i} family members or at least of the receptors to which they couple.

To reveal additional functions of G\textsubscript{i} family members, we have studied the effects G protein \alpha subunits on the activation of the low molecular weight GTPase Rap1B. Rap1 is the most closely related family member to Ras itself, and like other Ras family members, it is activated by the exchange of GTP for GDP. Activation of Rap1 has been shown to mediate activation of integrins on the surface of fibroblasts and lymphocytes (14–17) and has been proposed to activate \alpha\textsubscript{v}\beta\textsubscript{3} on the surface of platelets (18, 19). Platelets express Rap1B and Rap2B, but only Rap1B is activated within the rapid time frame required for a role in platelet activation (24). The role of Rap1 in platelet biology has been of interest for some years because of its high expression level and prominence as a substrate for phosphorylation by cAMP-dependent protein kinase (31). More recent studies have demonstrated that Rap1 is activated by agonists that stimulate intracellular calcium release in platelets as well as by treatment of platelets with calcium ionophores that allow entry of extracellular calcium (18, 24). In this report, we sought to establish that Rap1 may also be activated downstream of G\textsubscript{i} family members through a calcium-independent mechanism that may account in part for the ability of G\textsubscript{i} family members to contribute to platelet activation.

The present work and two recent studies (17, 18) support the view that Rap1 is rapidly activated by G\textsubscript{i} family members in platelets, providing a candidate signaling pathway that may explain the contribution of G\textsubscript{i} family members to platelet aggregation. We provide evidence using platelets from mice deficient in either G\textsubscript{i2} family members or G\textsubscript{i1}, that platelet agonists that stimulate G protein-coupled receptors activate Rap1 through G\textsubscript{i} dependent pathways. Activation of a G\textsubscript{i} coupled receptor also appears to be a required signal for activation of Rap1 induced by activation of the collagen receptor GPVI or by force clustering of the Fc\gammaRIIA receptor on platelets (20, 21).
Both of these receptors stimulate the release of calcium from intracellular stores but require release of ADP from dense granules upon platelet activation to generate a G_i-dependent signal. These data present an interesting parallel to the mechanism of thrombin-stimulated Rap1 activation, which is also largely dependent on activation of the G_i-coupled P2Y12 receptor by released ADP (the P2Y12 receptor antagonist MeSAMP or loss of G_{i2} strongly inhibits Rap1 activation by thrombin; data not shown; Fig. 3; see discussion below).

Initial evidence supporting the hypothesis that Rap1 activation may be induced by a calcium-independent pathway is that epinephrine, which does not elicit a detectable calcium response, is capable of inducing Rap1 activation, albeit weakly compared with other agonists (our data here; see also Ref. 20). We show here that this signal is dependent on the presence of the G_i family member G_{i2} because the ability of epinephrine to activate Rap1 is lost in platelets from G_{i2}/-/ mice. This is consistent with results showing that epinephrine-mediated potentiation of aggregation in response to various agonists is also dependent on G_{i2} (11). Similarly, the amount of Rap1 activated in response to ADP is severely diminished in mice deficient in G_{i2} but not in G_{i2}-/ or G_{i3}-/ - platelets, consistent with the expectation that the G_i-coupled ADP receptor, P2Y12, couples mainly to G_{i2}. It is interesting to note that in the G_{i3} knockout mouse, ADP-mediated Rap1 activation (like ADP-induced aggregation) is diminished but not abrogated. Taken together, these results suggest that the contribution of G_i signaling to ADP-stimulated Rap1 activation is at least as substantial as that of the calcium-mediated pathways stimulated by G_{i3}. In contrast, thrombin-stimulated Rap1 activation is partially diminished in the G_{i2}-/- mouse but is nearly absent in the G_{i3}-/- mouse. These data suggest that thrombin-stimulated Rap1 activation is due to both G_{q}-mediated and G_i-mediated signals (see Fig. 8). Stimulation of G_{i} by thrombin likely accomplishes two functions in this pathway: 1) G_{i}, through activation of protein kinase C, contributes to release of ADP from dense granules, which in turn activates both P2Y1 and P2Y12. P2Y12 activates G_{i2}, providing the G_i-dependent component of thrombin signaling to Rap1, and 2) G_{q} stimulates calcium release from platelet storage granules, leading to a calcium-mediated activation of Rap1 (Fig. 8). This is consistent with evidence suggesting that PAR4 (the sole known signaling receptor for thrombin in mouse platelets) does not couple directly to G_i family members and requires secondary release of ADP to stimulate G_i family members.

Activation of Rap1 by G_{q}-coupled agonists is presumably mediated by a Ca^{2+}-sensitive exchange factor (see below). There are several possible pathways by which G_i family members could regulate Rap1 activation and platelet reactivity. One possibility is that activation of G_i family members may depress basal levels of cAMP in the platelet cytosol, which may then increase the amount of calcium released in response to sub-threshold amounts of phospholipase C activation. However, although the absence of G_{i2} and G_{i3} diminishes Rap1 activation by epinephrine and ADP, respectively, platelets from mice lacking either of these G proteins display little or no difference in agonist-induced changes in cytosolic [Ca^{2+}]. These observations, together with the fact that we are unable to detect increases in Rap1 activation after addition of membrane-permeable adenylyl cyclase inhibitors, suggest that G_i-dependent Rap1 activation is not due solely to the effects of cAMP on calcium release. It should be noted, however, that the calcium

**Fig. 5.** Rap1 activation in the presence of ADP receptor inhibitors in mouse and human platelets. A and B, platelets were isolated as described and pretreated for 5 min with no inhibitor, 300 μM A3P5PS, or 30 nM MeSAMP. Platelets were then treated for an additional 5 min with 10 μM ADP or 10 μM epinephrine, and Rap-GTP was isolated. C, platelets were isolated and incubated with Fura-2AM as described. Cells were then pretreated for 5 min with either 300 μM A3P5PS or no antagonist and stimulated with 10 μM ADP or 0.1 unit/ml thrombin as indicated.
chelator BAPTA-AM abrogates Rap activation in response to either epinephrine or ADP (our own data not shown; Ref. 20). It is possible that the as yet unidentified GEF(s) or GAP(s) that mediate Rap activation in platelets require some basal level of calcium but are regulated by additional mechanisms as well.

A second mechanism by which G\textsubscript{i} family members could promote Rap1 activation in platelets is by inhibiting Rap1GAP, which accelerates the GTPase activity of Rap1, thereby inactivating it. Marti and Lapetina (32) have demonstrated a calcium-dependent Rap1GEF, such as CalDAG-GEF. Rap1 activation by G\textsubscript{i} family members appears to be dependent on PI3K but may also involve some isoform of Rap1GAP. These mechanisms remain to be established.

The amount of GTP-bound Rap1 in platelets can be increased by activating receptors coupled to G\textsubscript{i} or G\textsubscript{q} family members. Epinephrine binds to \(\alpha_{2A}\) adrenergic receptors, which activates Rap1 via a G\textsubscript{q2}-dependent pathway that is at least partially dependent on PI3K. ADP stimulates both G\textsubscript{i2}- and G\textsubscript{q}-coupled receptors, leading to two pathways of Rap1 activation: a calcium-dependent pathway downstream of G\textsubscript{q} and a G\textsubscript{i}-dependent pathway that appears to be dependent on activation of PI3K. Thrombin binds to either PAR1 and PAR3 on human platelets or PAR1 and PAR4 on mouse platelets and activates G\textsubscript{q} associated with PAR1 or PAR4. This results in release of ADP from dense granules, which in turn stimulates the ADP receptors P2Y1 and P2Y12. Thus, thrombin also stimulates Rap1 activation through both G\textsubscript{q} and G\textsubscript{i}-dependent pathways. The mechanisms of G protein-mediated Rap1 activation are not yet completely clear. G\textsubscript{q}-mediated increase of [Ca\textsubscript{2+}]\textsubscript{i} likely stimulates a calcium-dependent Rap1GEF, such as CalDAG-GEF. Rap1 activation by G\textsubscript{i} family members appears to be dependent on PI3K but may also involve some isoform of Rap1GAP. These mechanisms remain to be established.
Yet another mechanism that often mediates G\textsubscript{i}-dependent receptors is the activation of G\textsubscript{i} and G\textsubscript{q}-coupled receptors. A known effector of G\textsubscript{i}-coupled by subunits is the \(\gamma\) isoform of PI3K. Recent studies have shown that PI3K is rapidly phosphorylated by platelet agonists (36), that platelet aggregation stimulated by the G\textsubscript{i}-coupled ADP receptor P2Y12 can be inhibited by PI3K inhibitors (10), and that the lack of PI3K in mouse platelets reduces ADP-dependent platelet aggregation and fibrinogen binding (27). These reports led us to investigate if activation of PI3K might contribute to G\textsubscript{i}-dependent Rap1 activation. Indeed, activation of Rap1 elicited by epinephrine or ADP is sensitive to the PI3K inhibitors wortmannin and LY294002 (our results and those in Refs. 20 and 21). Platelets from mice lacking PI3K showed a variable decrease in agonist-stimulated Rap1 activation that reached significance only with ADP. Taken together with the inhibitor studies, this would seem to suggest that more than one isoform of PI3K is involved in the G\textsubscript{i}-mediated activation of Rap1. The positive identification of the Rap1-specific exchange factors and GTPase-activating proteins activated upon platelet stimulation should provide more complete information regarding the mechanisms of activation of Rap1 in platelets.

The hypothesis of our laboratory and others, that Rap1 is required for activation of integrin \(\alpha_{IIb}\beta_{3}\) by a number of agonists, including ADP and epinephrine, is under investigation pending reliable methods of inhibiting Rap1 activity in platelets. However, in the course of these studies, we have now observed three conditions that cause Rap1 activation but are insufficient to cause platelet aggregation: 1) treatment of platelets with a GST-fused extracellular domain of EphB1 to activate ephrin B1,\(^3\) 2) addition of epinephrine when applied to washed platelet preparations, and 3) low micromolar concentrations of ADP in the presence of the P2Y1 inhibitor A3P5PS. This probably implies that Rap1 activation alone is not sufficient to cause activation of the integrin \(\alpha_{IIb}\beta_{3}\). It is interesting that GST-epphB1, epinephrine, and ADP in the presence of A3P5PS do not elicit a calcium response; perhaps another calcium-dependent process in addition to Rap1 activation is required for integrin activation.

In summary, the studies in this article demonstrate a novel mechanism for the regulation of Rap1 activation downstream of the activation of G\textsubscript{i} family members and demonstrate a new downstream effector pathway for G\textsubscript{i} and G\textsubscript{q}. This mechanism is at least partially dependent on the presence of PI3K. We believe that Rap1 may play an integral role in the early events leading to platelet activation and that these studies provide a mechanism by which G\textsubscript{i} family members contribute to this process.

Acknowledgments—We thank Dr. Stefan Offermanns (University of Heidelberg, Heidelberg, Germany) for G\textsubscript{i} and \(\gamma\) mice and Dr. Dianaing Wu (University of Rochester, Rochester, NY) and Dr. Charles Abrams (University of Pennsylvania) for generating and propagating PI3K\textsubscript{γ−/−} mice and for helpful discussions.

\(^3\) Prevost, N., Woulfe, D., Tanaka, T., and Brass, L. F. (2002) \textit{Proc. Natl. Acad. Sci. U. S. A.}, in press.

REFERENCES

1. Woulfe, D., Yang, J., Prevost, N., O'Brien, P. J., and Brass, L. F. (2002) in \textit{Platelets (Michelson, A. D., ed), Academic Press, San Diego, CA}, in press.
2. Brass, L. F. (1999) \textit{J. Clin. Invest.} 104, 1663–1665
3. Offermanns, S., Toome, C. F., Hu, Y. H., and Simon, M. I. (1997) \textit{Nature} 389, 183–186
4. Gabberta, J., Yang, X., Kowalska, M. A., Sun, L., Dhanasekaran, N., and Ras, A. K. (1997) \textit{Proc. Natl. Acad. Sci. U. S. A.} 94, 8750–8755
5. Woulfe, D., Yang, J., and Brass, L. (2001) \textit{J. Clin. Invest.} 107, 1503–1505
6. Gagnon, A. W., Manning, D. B., Catani, L., Gewirtz, A., Ponzec, M., and Brass, L. F. (1991) \textit{Blood} 78, 1247–1253
7. Williams, A. G., Woulfe, D. M., Ponzec, M., Manning, D. B., Gewirtz, A. M., and Brass, L. F. (1990) \textit{Blood} 76, 721–730
8. Foster, C. J., Prosser, D. M., Agans, J. M., Zhai, Y., Smith, M. D., Lachowicz, J. E., Zang, P. L., Gustafson, E., Monzana, F. J., Jr., Wiekowski, M. T., Abbondandolo, S. J., Cook, D. N., Bayne, M. L., Lira, S. A., and Chintala, M. S. (2001) \textit{J. Clin. Invest.} 107, 1591–1598
9. Hillopolet, G., Jantzen, H. M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R. B., Nurden, P., Nurden, A., Julien, D., and Conley, P. B. (2001) \textit{Nature} 409, 202–207
10. Kauffenstein, G., Bergmeier, W., Eckly, A., Ohnmann, P., Leon, C., Czajewski, J. P., Niewiadat, B., and Gachet, C. (2001) \textit{FEBS Lett.} 505, 281–289
11. Yang, J., Wu, J., Kowalska, M. A., Dalvi, A., Prevost, N., O'Brien, P. J., Manning, D., Ponzec, M., Lucki, I., Blendy, J. A., and Brass, L. F. (2000) \textit{Proc. Natl. Acad. Sci. U. S. A.} 97, 9884–9889
12. Jantzen, H. M., Mileston, D. S., Gouessat, L., Conley, P. B., and Mortensen, R. M. (2001) \textit{J. Clin. Invest.} 108, 477–483
13. Daniel, J. L., Dangelmaier, C., Jin, J., Kim, Y. B., and Kunapuli, S. P. (1999) \textit{Thromb. Haemostasis} 82, 1322–1326
14. Reedquist, K. A., Ross, E., Koepp, A. A., Wollhuis, R. M., Zwartkruis, F. J., van Kooyk, Y., Salmon, M., Buckley, C. D., and Bos, J. L. (2000) \textit{J. Cell. Biol.} 148, 1311–1318
15. Tsukamoto, N., Hattori, M., Yang, H., Bos, J. L., and Minato, N. (1999) \textit{J. Biol. Chem.} 274, 18463–18469
16. Katagiri, K., Hattori, M., Minato, N., Irie, S., Takatoku, K., and Kinashi, T. (2000) \textit{Mol. Cell. Biol.} 20, 1856–1863
17. Sebdoua, E., Bracke, M., Tugel, T., Hogg, N., and Cantrell, D. A. (2002) \textit{Nat. Immunol.} 3, 251–258
18. Franko, B., Akkerman, J. W., and Bos, J. L. (1997) \textit{EMBO J.} 16, 252–259
19. Bertoni, A., Taladokoro, S., Eto, K., Pampori, N., Parise, L. V., and Brass, L. F. (2002) \textit{Proc. Natl. Acad. Sci. U. S. A.}, in press.
20. Lova, P., Paganini, S., Sinigaglia, F., Baldini, C., and Torti, M. (2002) \textit{J. Biol. Chem.} 277, 12009–12015
21. Larson, M. K., Chen, H., Kahn, M. L., Fabre, J.-E., Koller, B. H., Mortensen, R. M., Conley, P. B., and Parise, L. V. (2002) \textit{Blood} 98, 751a
22. Nagata, K., Ye, C., Jain, M., Mileston, D. S., Liao, R., and Mortensen, R. M. (2000) \textit{J. Biol. Chem.} 275, 82733–82740
23. Koziak, K., Kaczmarek, E., Park, S. Y., Fu, Y., Avraham, S., and Avraham, H. (2001) \textit{Br. J. Haematol.} 114, 134–140