Thrombin Receptor Activation and Integrin Engagement Stimulate Tyrosine Phosphorylation of the Proto-oncogene Product, p95\textsuperscript{vav}, in Platelets\textsuperscript{*}

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The vav proto-oncogene product, p95\textsuperscript{vav} or Vav, is primarily expressed in hematopoietic cells and has been shown to be a substrate for tyrosine kinases. Although its function is unknown, Vav shares a region of homology with DBL, an exchange factor for the Rho family of GTP-binding proteins. The presence of this domain and the observation that cells transformed with Vav display prominent stress fibers and focal adhesions similar to those that are observed in RhoA transformed cells suggest that Vav may play a role in regulating the actin cytoskeleton. We have, therefore, examined Vav phosphorylation in platelets, which undergo dramatic cytoskeletal reorganization in response to agonists. Two potent platelet agonists, thrombin (via its G protein-coupled receptor) and collagen (via its interaction with the α5β1 integrin), caused Vav to become phosphorylated on tyrosine. Weaker platelet agonists, including ADP, epinephrine and the thromboxane A2 analog, U46619, did not. The phosphorylation of Vav in response to thrombin was maximal within 15 s and was unaffected by aspirin, inhibitors of aggregation, or the presence of the ADP scavenger, apyrase. Vav phosphorylation was also observed when platelets became adherent to immobilized collagen (via integrin α2β1), fibronectin (via integrin α5β1), and fibrinogen (via integrin α1β3). These results show that Vav phosphorylation by tyrosine kinases 1) occurs during platelet activation by potent agonists, 2) also occurs when platelets adhere to biologically relevant matrix proteins, 3) requires neither platelet aggregation nor the release of secondary agonists such as ADP and TXA\textsubscript{2}, and 4) can be initiated by at least some members of two additional classes of receptors, G protein-coupled receptors and integrins, providing further evidence that both of these can couple to tyrosine kinases.

The biochemical function of Vav has been investigated extensively, but without complete resolution. The presence of a domain homologous to the catalytic region of the DBL protein suggests that Vav may have a function similar to DBL. DBL is a guanine nucleotide exchange factor for RhoA and Cdc42Hs, two members of the Rho family of low molecular weight GTP-binding proteins which are thought to regulate agonist induced cytoskeletal reorganization in fibroblasts. The possibility that Vav may also participate in cytoskeletal reorganization is suggested by its homology to DBL and by the observation that cells transformed with Vav display an increased number of stress fibers and focal adhesions, making them morphologically indistinguishable from cells expressing activated forms of DBL or Rho. However, to date, efforts to demonstrate that Vav has exchange activity for Rho family members have been unsuccessful. Instead, Gulbins et al. have reported that Vav possesses nucleotide exchange activity for Rac, an unexpected observation given that Vav does not contain the CDC25 homology domain present in other mammalian and yeast Rho exchange factors. However, reports describing an association between Vav and Grb-2 support the speculation that this activity may be attributable to an associated protein, rather than Vav itself. Taken together, these observations suggest that Vav could be involved in at least two different pathways involving low molecular weight GTP-binding proteins and may play a role in cytoskeletal organization. However, the precise nature of this role and the molecular basis for Vav's involvement still remain to be determined.

In the present study, we have examined the role of Vav in platelet activation. In response to a variety of extracellular agonists, platelets form multicellular aggregates, secrete the contents of their storage granules, and adhere and spread on extracellular matrix proteins. This process is characterized by extensive cytoskeletal reorganization and involves the loss of their initial discoid shape and the extension of lamellae and filopodia. These events are typically triggered by agonists that activate G protein-coupled receptors, such as thrombin, or by integrin-mediated adhesion of platelets to matrix proteins, such as collagen. Activation of platelets via these mechanisms has been shown to result in a dramatic increase in the tyrosine phosphorylation of multiple platelet proteins, only a few

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which have been identified (30). Given the evidence that Vav may participate in pathways leading to cytoskeletal reorganization, the present studies 1) examine whether Vav is one of the proteins that becomes tyrosine-phosphorylated during platelet activation, 2) define the types of receptors to which Vav phosphorylation is linked, and 3) characterize its phosphorylation during the process of platelet activation. The results show that in suspension two strong platelet agonists, thrombin and collagen, rapidly induce the phosphorylation of Vav. Phosphorylation occurs on tyrosine residues and does not require platelet aggregation or the release of secondary agonists such as ADP and TxA$_2$. The results also show that Vav becomes phosphorylated when platelets undergo integrin-mediated adhesion and spreading on collagen, fibrinogen, and fibronectin. Thus, in addition to characterizing Vav phosphorylation in the context of platelet activation, these results describe two additional classes of receptors, G protein-coupled receptors and integrins, whose activation can lead to the phosphorylation of Vav.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human α-thrombin (New York State Department of Health, Albany, NY) was synthesized as described below. ADP and epinephrine were obtained from Sigma. U46619 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Fab fragments of the monoclonal antibody 7E3 were provided by Dr. Barry Coler (Mt. Sinai Hospital, New York, NY) and Dr. Robert Jordan (Centocor) and an anti-human integrin β1 monoclonal antibody (ascites) was obtained from Life Technologies, Inc. Fab fragments of the LIBS6 antibody were provided by Dr. Mark Ginsberg (Scripps Institute). A rabbit polyclonal antisera directed toward residues 577–590 of the Vav protein was provided by Dr. Jose Bustelo and Dr. Mariano Barbacid (Bristol-Meyers Squibb). Anti-phosphotyrosine monoclonal antibodies 4G10 and PY 20 were provided by Dr. Tom Roberts (Dana Farber Cancer Institute) and obtained from ICN Biochemicals, Inc., respectively. Bovine serum albumin (BSA) was also obtained from ICN Biochemicals, Inc.

**Platelet Preparation and Activation**—Platelets were removed from plasma by filtration through Sephrose 2B and resuspended in medium containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 20 mM HEPES, pH 7.4, 0.1% glucose, 0.1% BSA at adjusted platelet concentration of 8 x 10$^5$ platelets/ml (31). Typically, 500 μl of platelets were activated in suspension by the addition of either 1 unit/ml thrombin, 50 μM SFLLRN, 30 μM collagen, 20 μM epinephrine, 20 μM ADP, or 5 μM U46619 while being stirred in a Chrono-log (Havertown, PA) aggregometer. When indicated, the platelets were incubated with aspirin (1 mM) for 30 min and aspirase (10 units/ml) for 5 min prior to agonist stimulation. To inhibit platelet aggregation, 20 μg/ml antibody 7E3, directed toward the β subunit of α$_2$-Ib/β$_3$, or 200 μM RGDS (Sigma) were added 20 min prior to agonist stimulation. The anti-β1 antibody was similarly added at a dilution of 1:100 to inhibit platelet activation by collagen.

**Platelet Adhesion and Spreading**—Platelets were removed from plasma by filtration through Sephrose 2B and resuspended in medium containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 20 mM HEPES, pH 7.4, 0.1% glucose, 0.1% BSA at adjusted platelet concentration of 8 x 10$^5$ platelets/ml (31). Typically, 500 μl of platelets were activated in suspension by the addition of either 1 unit/ml thrombin, 50 μM SFLLRN, 30 μM collagen, 20 μM epinephrine, 20 μM ADP, or 5 μM U46619 while being stirred in a Chrono-log (Havertown, PA) aggregometer. When indicated, the platelets were incubated with aspirin (1 mM) for 30 min and aspirase (10 units/ml) for 5 min prior to agonist stimulation. To inhibit platelet aggregation, 20 μg/ml antibody 7E3, directed toward the β subunit of α$_2$-Ib/β$_3$, or 200 μM RGDS (Sigma) were added 20 min prior to agonist stimulation. The anti-β1 antibody was similarly added at a dilution of 1:100 to inhibit platelet activation by collagen.

**Platelet Lysis and Immunoprecipitation**—Platelets were lysed by sonication in 5 mg/ml bovine serum albumin, 100 μM fibrinogen from Kabi (distributed by Helena Laboratories, Beaumont, TX), 25 μg/ml collagen (Collaborative Biomedical Products, Bedford, MA), or 50 μg/ml collagen. A 1 ml suspension of platelets was added to plates for 1 h, after which they were washed 3 times with phosphate-buffered saline, and adherent platelets were lysed as described below.

**Platelet Lysis and Immunoprecipitation**—Platelets activated in suspension were lysed with an equal volume of 2 x RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 10 mM Tris [pH 7.2], 1 mM phenylmethylsulfonyl fluoride, 1.0 mM Na$_2$VO$_4$, and aprotonin at 100 kallikrein inactivator units/ml) for 15 min. To maintain consistency, adherent platelets were lysed with a 1:1 mixture of the platelet resuspension buffer and 2 x RIPA buffer. Lysates were then clarified by centrifugation at 16,000 × g for 5 min. The supernatant was removed, and 5 μl of Vav antisera or control antisera was used to immunoprecipitate as described (31). Samples from adhesion experiments were normalized with respect to protein concentration prior to immunoprecipitation.

**RESULTS**

**Platelet Adhesion and Spreading**—Platelets were removed from plasma by filtration through Sephrose 2B and resuspended in medium containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 20 mM HEPES, pH 7.4, 0.1% glucose, 0.1% BSA at adjusted platelet concentration of 8 x 10$^5$ platelets/ml (31). Typically, 500 μl of platelets were activated in suspension by the addition of either 1 unit/ml thrombin, 50 μM SFLLRN, 30 μM collagen, 20 μM epinephrine, 20 μM ADP, or 5 μM U46619 while being stirred in a Chrono-log (Havertown, PA) aggregometer. When indicated, the platelets were incubated with aspirin (1 mM) for 30 min and aspirase (10 units/ml) for 5 min prior to agonist stimulation. To inhibit platelet aggregation, 20 μg/ml antibody 7E3, directed toward the β subunit of α$_2$-Ib/β$_3$, or 200 μM RGDS (Sigma) were added 20 min prior to agonist stimulation. The anti-β1 antibody was similarly added at a dilution of 1:100 to inhibit platelet activation by collagen.

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**Anti-phosphotyrosine Immunoblotting**—Anti-phosphotyrosine immunoblots were performed using a mixture of 4G10 and PY 20 antibodies, a goat anti-mouse immunoglobulin-horseradish peroxidase conjugated antibody (Bio-Rad Laboratories), and a chemiluminescence detection kit (Amersham) (31).

**Thrombin Rapidly Induces the Tyrosine Phosphorylation of Vav**

Thrombin rapidly induces the tyrosine phosphorylation of Vav in human platelets. To examine the phosphorylation state of Vav during platelet activation, platelets were incubated with 1 unit/ml thrombin for increasing amounts of time, after which the cells were lysed and Vav was immunoprecipitated using an antibody directed against residues 577–590 of the protein. As demonstrated by a Western blot with probe with an anti-phosphotyrosine antibody, Vav became phosphorylated within 15 s of thrombin addition and remained phosphorylated for at least 60 s (Fig. 1A). No detectable band was visible when normal rabbit serum was used for immunoprecipitation and the blots were probed with either the anti-phosphotyrosine antibody (lane 5, Fig. 1A) or an anti-Vav antibody (data not shown). At the high concentration of thrombin that was used, platelet aggregation was complete within 1 min. Extending the time to 2 min had no further effect on Vav phosphorylation (data not shown).

The cloned thrombin receptor is a G protein-coupled receptor that is activated when thrombin cleaves its N terminus, exposing a tethered ligand for the receptor (33, 34). Peptides corresponding to the first six residues of the tethered ligand (SFLLRN) have been shown to activate the receptor, mimicking many of the effects of thrombin (35, 36). In order to determine if the phosphorylation of Vav induced by thrombin was mediated by the cloned thrombin receptor, platelets were incubated with 50 μM SFLLRN. The peptide stimulated Vav phos-
phorylation with kinetics similar to thrombin (Fig. 1B).

Thrombin-induced Vav Phosphorylation Does Not Require Platelet Aggregation or the Secondary Agonists, ADP and TxA2—Thrombin stimulation of platelets leads to the release of two additional platelet agonists: thromboxane A2, generated from arachidonate via cyclooxygenase, and ADP, which is secreted from platelet dense granules. Both of these help to recruit platelets into a growing platelet plug. To determine whether released TxA2, and/or ADP is required for the observed induction of Vav phosphorylation, platelets were preincubated with aspirin, which irreversibly inhibits cyclooxygenase, and apyrase, which rapidly dephosphorylates ADP. In the experiment shown in Fig. 2, platelets were stimulated with thrombin in the absence (lane 2) or presence (lane 3) of aspirin and apyrase. Neither inhibitor affected Vav phosphorylation.

Much of the tyrosine phosphorylation observed in activated platelets has been reported to occur subsequent to platelet aggregation (30). Platelet aggregation is mediated by fibrinogen molecules which bind to αIIbβ3, cross-linking adjacent platelets. Aggregation can be inhibited by preincubating platelets with a monoclonal antibody, 7E3, directed toward αIIbβ3, or with the tetrapeptide, Arg-Gly-Asp-Ser (RGDS), both of which competitively inhibit fibrinogen binding (37, 38). Preincubation of platelets with either RGDS or a Fab fragment of 7E3 inhibited platelet aggregation (not shown), but had no effect on Vav phosphorylation, even in the presence of aspirin and apyrase (Fig. 2, lanes 4, 5, and 6). There was no Vav phosphorylation detected in platelets exposed to RGDS or 7E3 alone (data not shown). These results demonstrate that platelet aggregation is not required for thrombin-induced Vav phosphorylation.

Tyrosine Phosphorylation of Vav Is Induced by Thrombin and Collagen, but Not by Other Agonists—In order to determine if other platelet agonists that work through G protein-coupled receptors can cause Vav phosphorylation, we next stimulated platelets with either U46619, epinephrine, or ADP. U46619 activates platelet TxA2 receptors, epinephrine activates platelet α2-adrenergic receptors, and ADP is believed to activate an unidentified purinergic receptor (Fig. 3, A and B). Vav phosphorylation was not observed in response to any of these agonists, despite the fact that the experiment was performed under conditions in which the platelets were stirred and aggregation occurred. Thus, U46619, ADP, and epinephrine, even in the presence of aggregation, do not induce Vav phosphorylation.

Collagen was also tested for its ability to induce Vav phosphorylation when added to platelets in suspension. In contrast to U46619, ADP, and epinephrine, collagen stimulated Vav phosphorylation as well as, if not better than, thrombin (Fig. 3A, lanes 2 and 3). The major collagen receptor on the surface of human platelets is thought to be the αIIbβ3 integrin, also known as the glycoprotein Ia-IIa complex (39, 40), although other collagen receptors have been reported as well (41, 42). Preincubating the platelets with an anti-β3 antibody inhibited collagen-induced Vav phosphorylation (Fig. 3C) and platelet aggregation (not shown) by approximately 85%, suggesting that collagen stimulates both responses via its interaction with αIIbβ3. Fig. 4A shows the time course of Vav phosphorylation in platelets incubated with collagen. The phosphorylation occurred more slowly than it did in response to thrombin, as did the onset of platelet aggregation in response to collagen. Vav phosphorylation was detected within 1 min and was maximal at 3 min. As with thrombin, Vav phosphorylation in response to collagen was unaffected by aspirin and apyrase, which were present throughout this experiment, or by the addition of the anti-αIIbβ3 antibody, 7E3 (Fig. 4B).

Platelet Adhesion to Extracellular Matrix Proteins Induces Vav Phosphorylation—Since collagen mediates its effects on Vav via an integrin, we were interested in determining if other
integrins could also transduce a signal to Vav. Platelets have been reported to adhere to a number of extracellular matrix proteins through integrin receptors (43). To date, tyrosine phosphorylation has been reported as a consequence of platelet binding to immobilized collagen and fibrinogen. However, in the latter case, the phosphorylation of the proteins examined required the presence of released ADP (32). We examined the phosphorylated state of Vav after aspirin-treated platelets were allowed to adhere to immobilized collagen, fibrinogen, and fibronectin in the absence of a soluble agonist. In comparison to platelets exposed to immobilized albumin, to which the platelets did not adhere, adhesion to each of these extracellular matrix proteins resulted in the phosphorylation of Vav (Fig. 5). Aspirin, which was present throughout the experiment, and apyrase, which was added to the platelets in lanes 5–8, did not prevent Vav phosphorylation in platelets adherent to collagen or fibronectin suggesting that adhesion to these proteins alone is sufficient for Vav phosphorylation. However, apyrase greatly reduced the level of Vav phosphorylation observed in platelets adherent to fibrinogen (Fig. 5, lanes 3 and 7). Thus, platelet adhesion via the α2β1 and α5β3 integrins is sufficient to stimulate the phosphorylation of Vav while α1β3β3-mediated adhesion appears to require released ADP.

Adhesion to Fibrinogen, but Not Fibrinogen-mediated Aggregation, Induces Vav Phosphorylation—Fibrinogen binding to the surface of platelets in suspension normally occurs only after an agonist has activated the platelets, evoking a conformational change in α1β3β3 (43). Platelets that have not been activated can adhere to immobilized fibrinogen (44); however, adhesion results in the release of ADP, which can then switch the α1β3β3 complex to its “activated” state (32). One possible explanation for the inhibition of Vav phosphorylation by apyrase observed when platelets were added to fibrinogen-coated plates (Fig. 5) is that released ADP is supporting the Vav phosphorylation indirectly by activating α1β3β3. To test this hypothesis, we activated α1β3β3 in an agonist-independent manner by incubating the platelets with Fab fragments of LIBS6. LIBS6 is an antibody that binds to the integrin β3 subunit, inducing a conformational change in the α1β3β3 complex equivalent to its activated state (38). In the experiment in Fig. 6A, platelets were preincubated with LIBS6 and then plated on immobilized fibrinogen in the presence of apyrase.

Under these conditions, Vav became phosphorylated to an even greater extent than that observed when platelets were allowed to adhere to immobilized fibrinogen in the absence of apyrase. LIBS6 had no effect on platelets in suspension (see below). These results suggest that adhesion to fibrinogen, mediated by the “activated” form of α1β3β3 is sufficient to cause Vav phosphorylation.

In the presence of LIBS6, platelets in suspension can bind fibrinogen and aggregate. Fibrinogen binding in this manner has been reported to induce the phosphorylation of several 50–70-kDa proteins, a 140-kDa protein, and the tyrosine kinase, Syk (45, 46). We therefore tested the ability of LIBS6 and soluble fibrinogen to induce Vav phosphorylation in platelets in suspension. LIBS6-mediated fibrinogen binding and aggregation in this manner resulted in the phosphorylation of the previously described proteins (Fig. 6B, left); however, Vav was not phosphorylated (Fig. 6B, right). These results make the important distinction that adhesion to immobilized fibrinogen, but not fibrinogen-mediated aggregation, induces Vav phosphorylation. These results are consistent with the lack of Vav phosphorylation observed in aggregated platelets in the presence of U46619, ADP, and epinephrine.

**DISCUSSION**

At sites of vascular injury, platelets are exposed to collagen and other components of the damaged vessel wall in addition to locally generated thrombin and secreted products such as ADP and TXA2. In response to these agents, platelets adhere to the subendothelium and form multicellular aggregates. Studies performed in vitro have shown that platelet activation, adhesion, and aggregation are associated with tyrosine phosphorylation of multiple platelet proteins. In order to elucidate mechanisms of signal transduction in platelets, we and others have attempted to identify the proteins that are phosphorylated on tyrosine during platelet activation. In this report, we have examined the phosphorylation of Vav, a protein that may participate in the extensive cytoskeletal reorganization that occurs during platelet activation.

G Protein-coupled Receptor-mediated Tyrosine Phosphorylation—Thrombin, a G protein-coupled receptor agonist, is a potent activator of platelets that initiates the tyrosine phosphorylation of multiple proteins (30). The results presented in this report demonstrate that Vav is one of these. Vav phosphorylation occurred within 15 s of thrombin addition, was not dependent on the release of the secondary agonists ADP and TXA2, and appeared to be mediated by the known thrombin receptor since it also occurred when platelets were activated with the agonist peptide, SFLLRN. In contrast, other platelet agonists thought to activate G protein-coupled receptors, such as...
Vav Phosphorylation in Platelets

Fig. 6. High affinity adhesion to fibrinogen, but not fibrinogen-mediated platelet aggregation, induces Vav phosphorylation. A, aspirin-treated platelets were incubated with buffer (lanes 1 and 2) or 10 units/ml apyrase (lanes 3 and 4) for 5 min. Next, 150 μg/ml anti-LIBS6 Fab was added (lane 4) for an additional 5 min. Platelets were then incubated for 1 h on plates coated with BSA or fibrinogen as indicated. Vav immunoprecipitates were prepared as described in the legend to Fig. 5, and an immunoblot was probed with an anti-phosphotyrosine antibody. B and C, aspirin- and apyrase-treated platelets in suspension were incubated with 250 μg/ml fibrinogen for 5 min and then 150 μg/ml LIBS6 Fab or 30 μg/ml collagen, as indicated, for an additional 5 min while platelets were being stirred. Platelet lysates were prepared, and sequential immunoprecipitations were performed, first using an anti-Vav antibody (C) and then a polyclonal anti-phosphotyrosine antibody (B). Immunoblots were probed with an anti-phosphotyrosine antibody.

as U46619, epinephrine, and ADP, did not induce Vav phosphorylation in platelets, showing that there is selectivity in this particular response.

The phosphorylation on tyrosine residues of some platelet proteins in response to thrombin has been characterized as "aggregation independent," while other proteins are phosphorylated in a manner dependent on platelet aggregation. The present study shows that Vav can be placed in the former group of phosphorylated substrates. Taken together, the data suggest that Vav tyrosine phosphorylation is a direct consequence of thrombin receptor activation and does not require a secondary activation process involving released agonists or aggregation. Since the thrombin receptor, like other G protein-coupled receptors, does not possess intrinsic kinase activity, these results provide evidence that G protein-coupled receptors can activate tyrosine kinases. Accumulating data from platelet studies support this conclusion. p21ras, GAP and cortactin are both phosphorylated in response to thrombin in an aggregation-independent manner in platelets (31, 47). Similarly, thrombin induces the activation of the protein tyrosine kinases Src, Syk, and Jak2 (46-50). Several examples of G protein-coupled receptor-mediated tyrosine phosphorylation in cells other than platelets have also been described (51-54), and studies utilizing tyrosine kinase inhibitors have demonstrated a functional requirement for tyrosine kinases in G protein-coupled receptor-mediated potassium channel regulation (54), smooth muscle contraction (55), stress fiber formation (56, 57), and platelet activation (58-60). Taken together, these observations describe an emerging role for tyrosine phosphorylation in G protein-coupled receptor-mediated signal transduction. However, the mechanism by which G proteins induce tyrosine kinase activation still remains to be defined.

Responses to Integrin Engagement—Integrins, which are largely responsible for the adhesive properties of platelets, have also been shown to mediate protein tyrosine phosphorylation following their engagement by extracellular matrix proteins (61). In this study, we demonstrate that collagen induces the tyrosine phosphorylation of Vav in platelets. Furthermore, the phosphorylation was inhibited by an anti-β3 antibody, consistent with earlier reports that the α6β1 integrin plays a role in platelet activation by collagen (39, 40). Phosphorylation occurred when platelets were exposed to collagen in suspension or when allowed to adhere to immobilized collagen matrix. As in thrombin-treated platelets, this process did not require the release of ADP or TxA2. Interestingly, adhesion to extracellular matrix proteins, including fibrinogen and fibrinectin, also caused Vav phosphorylation. Platelet adhesion to each of these proteins has been described previously to be mediated by the integrins αIIbβ3 and αIIbβ1, respectively, although tyrosine phosphorylation has only been reported after adhesion of platelets to fibrinogen (32). This study demonstrates that multiple integrin receptors are capable of inducing Vav phosphorylation in platelets and that, with the exception of platelet adhesion to fibrinogen via αIIIbβ3, Vav phosphorylation does not require additional agonists. In the case of αIIbβ3, released ADP appears to be necessary to induce a conformational change in the integrin, rather than to serve as a secondary signal for Vav phosphorylation, since direct activation of αIIbβ3 by the LIBS6 antibody caused adhesion to fibrinogen and Vav phosphorylation even in the presence of apyrase. In this respect, Vav phosphorylation differs from the phosphorylation of FAK and p95/97, which have been described previously to require additional agonists as well as integrin engagement (62, 63). Notably, differences in signaling mediated by the activated versus unactivated form of the fibrinogen receptor have been reported previously (64).

Unexpectedly, adhesion to fibrinogen, in the presence of ADP released from intracellular stores, fully induced Vav phosphorylation, while platelet aggregation, even in the presence of exogenous ADP, did not. Both events involve fibrinogen binding mediated by the activated form of αIIbβ3 and in previous reports have been demonstrated to induce the tyrosine phosphorylation of similar proteins (32, 62). It is possible that the differences observed with Vav phosphorylation are due to the context of fibrinogen as it binds its receptor. These results, however, demonstrate a clear difference in signaling initiated by platelet aggregation versus adhesion and perhaps reflect a mechanism for mediating differential signals in response to cell-cell versus cell-matrix interactions.

Integrins have been demonstrated to regulate a number of cellular processes including cytoskeletal reorganization, gene expression, differentiation, and cell survival (61). Furthermore, tyrosine phosphorylation has been observed in numerous cell types following integrin engagement and therefore has been proposed to play a role in mediating these signals (61). Given
that Vav is expressed in hematopoietic cells other than platelets, where integrins are critical for processes such as leukocyte homing and activation (65), it will be interesting to determine if Vav also participates in these integrin-dependent functions. Lymphocytes from vav (−−)/RAG-2 (−−) chimeric mice should be useful in assessing this possibility.

Vav-mediated Signal Transduction in Platelets—Platelets contain a number of tyrosine kinases that could potentially be responsible for phosphorylating Vav, including Syk. Katzav et al. (66) have reported that ZAP-70, a kinase related to Syk, associates with Vav in T cells, suggesting that it may be the kinase responsible for Vav phosphorylation in that system. However, two observations suggest that this is not the case in platelets. First, our attempts to identify Syk or any other kinases in Vav immunoprecipitates from activated platelets, like those of Bustelo and Barbacid (13) in B cells, have been unsuccessful. Second, since the LB165 antibody induces Syk activation (46), but not Vav phosphorylation in nonadherent platelets, the activation of Syk in platelets does not completely correlate with Vav phosphorylation. Taken together, these observations suggest that Syk is not the kinase which phosphorylates Vav in platelets, although it is difficult to completely rule out this possibility during activation of platelets by all mechanisms.

The observation that Vav becomes phosphorylated in response to platelet agonists suggests that it may play a role in platelet activation. We initially began examining Vav in platelets due to its homology with the Rho exchange factor, DBL (18). Microinjection studies utilizing an activated variant of Rho and C3 exoenzyme, which inhibits Rho activity, have indicated that Rho is involved in the formation of focal adhesions and stress fibers (21, 22). Rho is also thought to play a role in platelet activation, as C3 exoenzyme inhibits platelet aggregation (67), although its effects on platelet adhesion and spreading have not been reported. If Vav does regulate Rho or a Rho-related protein, an interesting model emerges whereby thrombin and integrins could transduce a signal to Rho through the tyrosine phosphorylation of Vav, which in turn may affect the cytoskeletal reorganization that occurs during platelet activation. Notably, the agonists which induce Vav phosphorylation in platelets, the activation of Syk in platelets does not completely correlate with Vav phosphorylation. Taken together, these observations suggest that Syk is not the kinase which phosphorylates Vav in platelets, although it is difficult to completely rule out this possibility during activation of platelets by all mechanisms.

In summary, we have demonstrated that thrombin treatment and integrin-mediated adhesion induce the tyrosine phosphorylation of Vav in platelets. These receptors represent two additional classes of receptors which couple to Vav. It will be interesting to determine if G protein-coupled receptors and integrins in other cell types will also have an effect on Vav phosphorylation and Vav-mediated signaling. Additionally, our findings support the emerging role for tyrosine kinases in G protein-coupled receptor and integrin-mediated signal transduction. Given Vav’s homology to the DBL protein, and the correlation between the phosphorylation state of Vav and the dramatic cytoskeletal reorganization which occurs during platelet activation, perhaps Vav may participate in mediating this process. A better understanding, however, of the biochemical function of Vav is critical for determining the role of Vav in platelet function.

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