

Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets

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The ability of activated platelets to adhere to each other at sites of vascular injury depends on the integrin $\alpha_{IIb}\beta_3$. However, as aggregation continues, other signaling and adhesion molecules can contribute as well. We have previously shown that human platelets express on their surface the Eph receptor kinases EphA4 and EphB1 and the Eph kinase ligand ephrinB1. We now show that EphA4 is physically associated with $\alpha_{IIb}\beta_3$ in resting platelets, increases its surface expression when platelets are activated, and colocalizes with $\alpha_{IIb}\beta_3$ at sites of contact between platelets. We also show that Eph/ephrin interactions can support the stable accumulation of platelets on collagen under flow and contribute to postengagement "outside-in" signaling through $\alpha_{IIb}\beta_3$ by stabilizing platelet aggregates and facilitating tyrosine phosphorylation of the β_3 cytoplasmic domain. β_3 phosphorylation allows myosin to bind to $\alpha_{IIb}\beta_3$ and clot retraction to occur. The data support a model in which the onset of aggregation permits Eph/ephrin interactions to occur, after which signaling downstream from ephrinB1 and its receptors favors continued growth and stability of the thrombus by several mechanisms, including positive effects on outside-in signaling through $\alpha_{IIb}\beta_3$.

Thrombus formation is a process by which freely circulating platelets are activated by collagen or thrombin, adhering first to the injured vessel wall and then to each other, concomitant with the establishment of a fibrin clot. The formation of a thrombus can be an appropriate response to vascular injury or a pathological response to disease. In either setting, the recruitment of platelets into an expanding thrombus is normally supported by soluble agonists, such as thromboxane A₂ and ADP, which, along with thrombin and collagen, initiate the signaling events that activate the integrin $\alpha_{IIb}\beta_3$ (1). The activated integrin then binds to fibrinogen or von Willebrand factor, bringing platelets into contact with each other. How such contacts are maintained once they are formed is not fully understood, but increasing evidence suggests that the boundaries between adjacent platelets, like those between epithelial cells, are populated by signaling and adhesion molecules that help to sustain platelet activation and cement contacts between platelets (2). Some of the contact-dependent signaling is mediated by the integrins themselves (3), but other candidates exist as well, including the Eph kinases and their ligands, known as ephrins.

Although Eph kinases and ephrins are best known for their role in neuronal organization (4, 5) and as markers distinguishing arteries from veins during vasculogenesis (6), we have shown that human platelets express two Eph kinases, EphA4 and EphB1, and at least one ligand, ephrinB1 (7, 8). The Eph kinases are a large family of receptor tyrosine kinases with an extracellular ligand binding domain and an intracellular tyrosine kinase domain. The Eph B subfamily is distinguished from the Eph A subfamily by an insertion within the extracellular domain that helps to define the ligand preferences for the receptor (9). Ephrins are membrane-attached molecules that fall into two groups based on their mode of attachment. Ephrin A family members have a glycosphosphatidylinositol anchor. Ephrin B family members have a transmem-

brane domain. Ephrin A family members typically bind to Eph A kinases, and ephrin B family members usually bind to Eph B kinases, but exceptions do exist, including EphA4, which can bind to B and A ephrins. The ephrin B cytoplasmic domain includes sites for tyrosine phosphorylation and a docking site for proteins with PDZ domains. These sites give the B ephrins at least two ways to engage in protein-protein interactions (4, 10). As a result, when cells expressing ephrins contact cells expressing Eph kinases, bidirectional signaling can occur (11).

Although the full range of responses mediated by Eph kinases and ephrins remains to be described, it has been shown that Eph/ephrin interactions can have biologically meaningful effects on cell migration and adhesion. In the developing central nervous system, interactions between ephrins and Eph kinases help to establish boundaries and prevent inappropriate interactions (12). Deletion of genes encoding ephrins and Eph kinase can affect neurological development in model organisms, and mutations in ephrins and Eph kinases have been associated with human neurological disorders (12–14). Loss of ephrinB2 or EphB2 affects development of the vasculature (15). In some cases, the effects of Eph/ephrin interactions on migration and adhesion have been linked to effects on integrin engagement which, in different cells and different settings, can be inhibited or promoted (16–21). In the case of platelets, Eph/ephrin interactions favor cohesion: Forced clustering of ephrinB1 or Eph kinases promotes adhesion and aggregation, whereas inhibition of Eph/ephrin interactions leads to premature disaggregation at low agonist concentrations (7, 8). These effects appear to be partially due to the ability of ephrinB1 to activate Rap1 (8), a Ras family member that supports integrin activation in platelets (22). In addition, in activated platelets, EphA4 becomes associated with the Src-related kinases Fyn and Lyn (7), an association that is relevant for the present studies.

Contacts between circulating platelets are normally transient, but the onset of integrin engagement permits Eph/ephrin interactions to occur as well. In the present studies, we have examined the interrelationships that occur between Eph kinases and integrins in platelets by examining the physical and functional associations that develop between $\alpha_{IIb}\beta_3$, EphA4, and ephrinB1 during platelet activation. The results show that EphA4 is constitutively associated with $\alpha_{IIb}\beta_3$ in platelets and, like $\alpha_{IIb}\beta_3$, shows an increase in surface expression when platelets are activated. The results also show that, in addition to promoting Rap1 activation, Eph/ephrin interactions support the stable accumulation of platelets on collagen under flow

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Abbreviation: PMA, phorbol myristate acetate.

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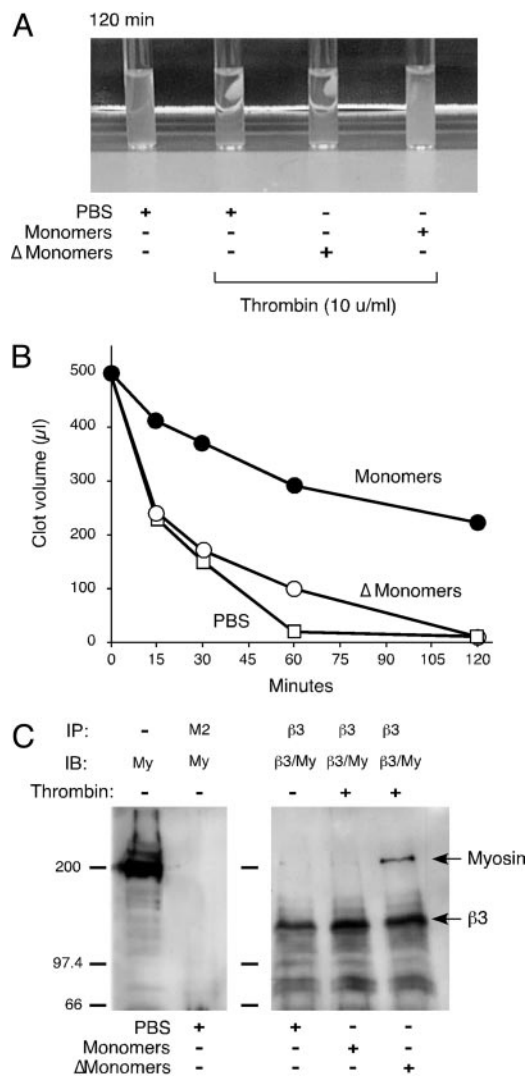


Fig. 1. Inhibition of Eph/ephrin interactions impairs clot retraction and the association of myosin with $\alpha_{IIb}\beta_3$. (A and B) Platelet-rich plasma was preincubated for 10 min with either PBS, 10 μ g/ml His-EphA4 plus His-EphB1 (Monomers), or 10 μ g/ml binding-domain-deleted His- Δ EphA4 plus His- Δ EphB1 (Δ Monomers), after which 5–10 units/ml thrombin was added to initiate thrombus formation. The clots were photographed after 120 min, and clot volume was determined at each time indicated. (C) Platelets were incubated for 10 min with thrombin. Proteins were immunoprecipitated (IP) with anti- β_3 or an isotype-matched control (M2) and then immunoblotted (IB) with anti- β_3 plus anti-myosin (My). The experiments shown are representative of three similar studies.

Myosin-binding requires the phosphorylation of two Tyr residues (Tyr-773 and Tyr-785) within the β_3 cytoplasmic domain (28). An elegant demonstration of the requirement was obtained in a mouse model in which substitution of Phe for Tyr-773 and Tyr-785 (Tyr-747 and Tyr-759 in mice) prevented myosin binding and impaired clot retraction (31), much as we observed in human platelets when Eph/ephrin interactions were blocked. To determine whether Eph/ephrin interactions play a role in β_3 phosphorylation, platelets were incubated with an agonist or with GST-EphB1, a fusion protein comprised of the extracellular domain of EphB1 joined to GST that can induce clustering and activation of ephrinB1 in platelets (7, 8). Afterward, the platelets were lysed, β_3 was immunoprecipitated, and the precipitate was probed with an antibody specific for β_3 phosphorylated on Tyr-773 (pTyr-773- β_3). Each of the agonists caused β_3 phosphorylation, as did GST-EphB1

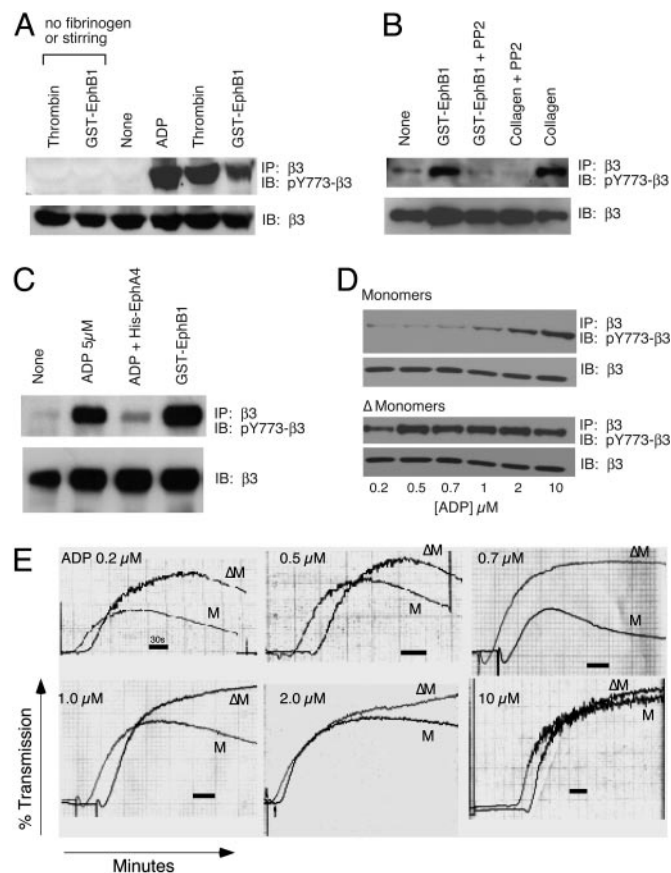


Fig. 2. Tyr phosphorylation of the β_3 cytoplasmic domain. Except where indicated, platelets were incubated with an agonist for 10 min under aggregating conditions (300 μ g/ml fibrinogen plus stirring), then lysed, immunoprecipitated with anti- β_3 , and immunoblotted with a phospho-specific antibody to β_3 Tyr-773 (pTyr-773- β_3). (A) ADP (10 μ M), thrombin (1 units/ml), or GST-EphB1 (16 μ g/ml). (B) GST-EphB1 (16 μ g/ml) or collagen (100 μ g/ml) with or without the Src family inhibitor PP2 (10 μ M). (C) ADP (5 μ M), His-EphA4 (20 μ g/ml), or GST-EphB1 (16 μ g/ml). (D) ADP dose-response curve for β_3 phosphorylation preincubated for 10 min with 10 μ g/ml His-EphA4 plus His-EphB1 (denoted Monomers or M) or His- Δ EphA4 plus His- Δ EphB1 (Δ Monomers or Δ M). (E) The aggregation traces for the samples analyzed in D.

(Fig. 2). Phosphorylation required fibrinogen and stirring and was inhibited by the Src family inhibitor PP2 or by blocking Eph/ephrin interactions (Fig. 2B, C, and D). As reported in refs. 7 and 8, for platelet aggregation (and demonstrated again in Fig. 2E), the inhibition of phosphorylation produced by Eph/ephrin blockade was most evident at low agonist concentrations and less evident at high concentrations, suggesting that more than one mechanism is active in the phosphorylation of β_3 (see Discussion).

The Association of EphA4 with $\alpha_{IIb}\beta_3$. We next asked whether integrins and Eph kinases are physically and functionally associated in platelets. Flow cytometry with a monoclonal antibody directed at the EphA4 extracellular domain showed that EphA4 is detectable on the surface of resting platelets and, like $\alpha_{IIb}\beta_3$, increases in expression during platelet activation (Fig. 3A). The increase in EphA4 expression is not due to recruitment from α -granule membranes. Cell fractionation studies in which platelet lysates produced by nitrogen cavitation were resolved on Percoll gradients showed EphA4 in the membrane fraction in resting and activated platelets, as opposed to the α -granule protein P-selectin, which was in the granule fraction in resting platelets but moved to the membrane fraction when platelets were activated (Fig. 3B). Fluorescence

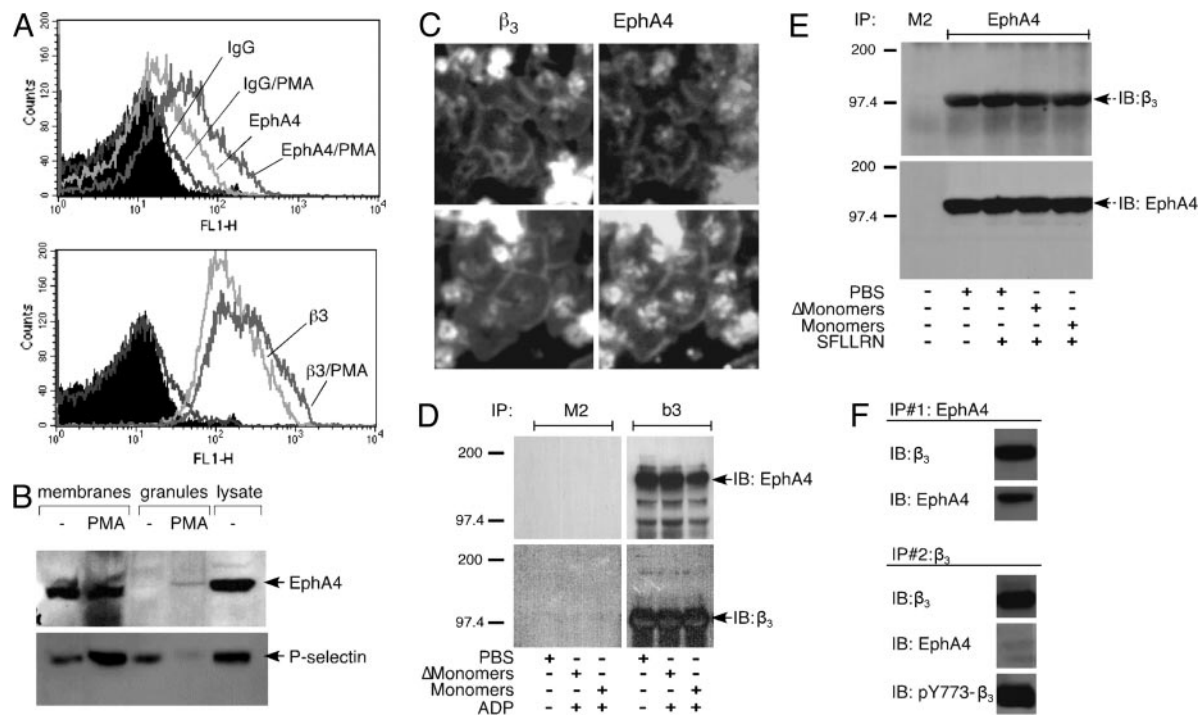


Fig. 3. Association of EphA4 with $\alpha_{IIb}\beta_3$. (A) Flow cytometry of platelets performed with monoclonal anti-EphA4 (clone 16c11) and anti- β_3 (clone SSA6). The results shown are representative of three similar studies. (B) Platelet lysates were resolved by Percoll gradient centrifugation into granule and membrane fractions. The results shown are representative of two similar studies. (C) Platelets were activated with 100 nM PMA and stained with anti-EphA4 or anti- β_3 . The experiment shown is representative of two similar studies. (D and E) Platelets were incubated for 5 min with 20 μ M ADP or 2 μ M SFLLRN under aggregating conditions in the presence of PBS, 10 μ g/ml His-EphA4 plus His-EphB1 (Monomers), or 10 μ g/ml binding-domain-deleted His- Δ EphA4 plus His- Δ EphB1 (Δ Monomers), then immunoprecipitated (IP) with anti- β_3 , anti-EphA4, or (as a negative control) antibody M2 and immunoblotted (IB). The experiment shown is representative of three similar studies. (F) Lysates were prepared from platelets activated with 100 nM PMA for 30 min, immunoprecipitated with anti-EphA4 (IP#1), and probed for β_3 and EphA4. Afterward, the supernatant from the first immunoprecipitate was reprecipitated with anti- β_3 (IP#2) and probed for β_3 , EphA4, and phospho- β_3 . The results shown are representative of two similar studies.

microscopy showed that EphA4 and β_3 have a similar distribution on the surface of activated platelets (Fig. 3C). Immunoprecipitation studies showed that EphA4 coprecipitated with β_3 by using an anti- β_3 antibody or an anti-EphA4 antibody (Fig. 3D and E). This association was observed in resting and activated platelets, and it persisted even when Eph/ephrin interactions were blocked, in contrast with the association between EphA4 and the Src family members Lyn and Fyn, which occurs only in activated platelets and only when Eph/ephrin interactions are permitted (7).

Although β_3 and EphA4 coprecipitated, the flow cytometry data suggest that the integrin is more abundant on the platelet surface than the kinase (Fig. 3A). The precise stoichiometry has not been reported, but it is likely that only some of the β_3 in platelets becomes phosphorylated during platelet aggregation. To determine whether the fraction of β_3 that becomes phosphorylated is restricted to the fraction of $\alpha_{IIb}\beta_3$ that remains associated with EphA4, the supernatant left behind after an initial immunoprecipitation with anti-EphA4 (IP #1 in Fig. 3F) was reprecipitated with anti- β_3 (IP #2), and the resulting precipitate was probed for β_3 , phospho- β_3 , and EphA4. The results show that there is residual β_3 in the anti-EphA4 supernatant that is not associated with EphA4 and that some of this “free” β_3 is phosphorylated. Assuming that this result is not simply due to a partial dissociation of $\alpha_{IIb}\beta_3$ /EphA4 complexes when the platelets were lysed, the presence of phospho- β_3 that does not coprecipitate with EphA4 could reflect movement of the integrin in and out of complexes with EphA4 during platelet activation. Alternatively, free phospho- β_3 could be because more than one mechanism exists for causing β_3 phosphorylation during platelet activation, with Eph/ephrin signaling being most relevant at sites where contact between platelet allows Eph/ephrin interactions to

occur and less relevant at sites where fibrinogen binding has occurred but platelet–platelet contact has not.

Thrombus Growth Under Arterial Flow Conditions. In addition to facilitating the binding of myosin to $\alpha_{IIb}\beta_3$, Tyr phosphorylation of the cytoplasmic domain of β_3 makes possible the binding of signaling and adaptor molecules whose subsequent contribution to intracellular events promotes thrombus growth and stability (3). Therefore, by modulating outside-in signaling, Eph/ephrin interactions might impact these events as well. To determine the effects of Eph/ephrin interactions on developing thrombi, fluorescently labeled human platelets were perfused over a collagen-coated surface at arterial flow rates. The growth of platelet thrombi was observed, and mean thrombus volume was determined at several points in the flow chamber by addition of the corresponding cross sectional areas in successive confocal planes. The results show that mean thrombus volume decreased by 40% when Eph/ephrin interactions were blocked (Fig. 4).

Discussion

Platelets are freely circulating blood cells that normally adhere to each other only at sites of vascular injury. Although injury can occur anywhere, the contribution of platelets to hemostasis is most critical in the arterial circulation, where rapid blood flow and ever-varying shear forces tend to oppose or destabilize hemostatic plug formation by pulling platelets away from each other and preventing the local accumulation of soluble agonists. Therefore, the ability of activated platelets to remain in contact with each other depends on the strength of the contacts between platelets, including the ability of integrins to remain engaged with their ligands and new adhesive

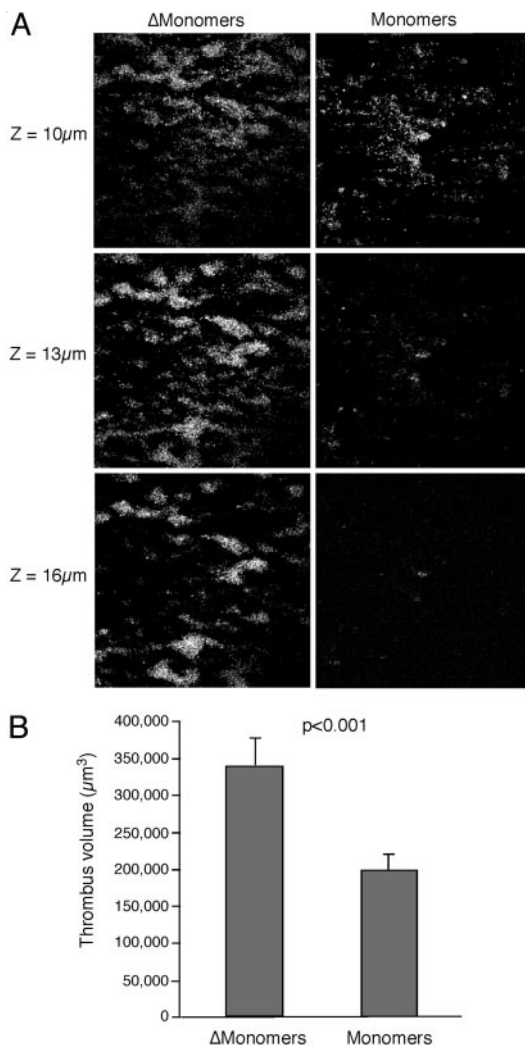


Fig. 4. Thrombus formation under arterial flow conditions. Mepacrine-labeled platelets in whole blood were perfused across a collagen-coated slide at a shear rate of $1,500 \text{ s}^{-1}$ in the presence of $10 \mu\text{g/ml}$ His-EphA4 plus His-EphB1 (Monomers) or $10 \mu\text{g/ml}$ binding-domain-deleted His- Δ EphA4 plus His- Δ EphB1 (Δ Monomers). After 5 min, the cells were fixed, and the slide was examined by confocal microscopy to determine thrombus volume. (A) Representative cross sections of the accumulated platelet thrombi in a series of parallel planes 10, 13, and $16 \mu\text{m}$ above the slide surface. (B) Average thrombus volume measured at four surface locations in each of four different experiments (mean \pm SEM).

and signaling interactions to occur once platelets come into contact. The present studies examine the contribution of ephrins and Eph kinases to this process. Previous work in cells other than platelets has shown that Eph/ephrin interactions can affect cell migration and adhesion by affecting integrin engagement. Depending on the cell and the context, Eph/ephrin engagement can promote or inhibit integrin activation (16–21). As a result, cells can move closer together when ephrins engage Eph kinases, or they can move apart. This process helps to establish boundaries within the developing central nervous system, as well as promoting coordinate development of the vasculature.

In the case of platelets, the evidence shows that Eph/ephrin interactions favor cohesion: Forced clustering of ephrinB1 or Eph kinases promotes adhesion and aggregation, whereas inhibition of Eph/ephrin interactions leads to premature disaggregation at low agonist concentrations (7, 8). The present studies suggest that, in addition to facilitating the inside-out signaling events that lead to irreversible integrin engagement, Eph/ephrin interactions play a

previously unappreciated role in integrin outside-in signaling by contributing to the phosphorylation of Tyr residues within the β_3 cytoplasmic domain. In turn, β_3 phosphorylation affects the binding of cytoplasmic proteins to $\alpha_{\text{IIb}}\beta_3$ and supports postaggregation events, such as clot retraction. The biological relevance of these events is reflected by studies showing that mutation of the Tyr residues in the β_3 cytoplasmic domain produces a phenotype in mice in which platelet aggregation becomes reversible at lower agonist concentrations, clot retraction is inhibited, and thrombus stability is impaired (31), phenotypes similar to those we have observed with human platelets when Eph/ephrin interactions are inhibited (ref. 7 and this study).

The ability of Eph/ephrin interactions to affect integrin engagement and integrin signaling shows that there is a functional relationship between $\alpha_{\text{IIb}}\beta_3$ and Eph kinases expressed in platelets. The present studies show that there is a physical relationship as well. Like $\alpha_{\text{IIb}}\beta_3$, EphA4 is broadly distributed on the platelet surface and can be found at the sites of contact between activated platelets. The surface expression of both molecules increases when platelets are activated, presumably because of recruitment from the surface connecting membrane system, and the two molecules coprecipitate from lysates of resting or activated platelets. These findings suggest that there is a constitutive association between the integrin and the Eph kinase. The data in the present studies do not establish whether EphA4 interacts directly or indirectly with $\alpha_{\text{IIb}}\beta_3$, and it is likely that the relationship between them is complex. The exact number of copies of EphA4 per platelets has not been determined, but the FACS data suggest that it is likely to be less than $\alpha_{\text{IIb}}\beta_3$, which is expressed at very high density. The reprecipitation experiments in Fig. 3 show that, at any given time, some of the $\alpha_{\text{IIb}}\beta_3$ is not associated with EphA4 and that unassociated $\alpha_{\text{IIb}}\beta_3$ includes some of the phospho- β_3 . Whether the presence of phosphorylated β_3 that is not associated with EphA4 reflects the existence of more than one mechanism for producing phosphorylated $\alpha_{\text{IIb}}\beta_3$ or whether it is because $\alpha_{\text{IIb}}\beta_3$ moves in and out of multimolecular complexes that include EphA4 remains to be determined.

Taken together, these observations suggest the following model. When platelets are activated by agonists, such as collagen, thrombin, or ADP, rapid signaling through their respective receptors initiates platelet aggregation by activating $\alpha_{\text{IIb}}\beta_3$, which binds to fibrinogen or von Willebrand factor, forming bridges between platelets. These bridges bring platelets into sustained contact with each other, allowing ephrinB1 on the surface of one platelet to engage its receptors on an adjacent platelet. Eph/ephrin interactions contribute another high-affinity interaction between the platelets and cause signaling complex formation that appears to have a number of consequences. One known consequence is helping to sustain Rap1B activation and, therefore, integrin engagement. Another consequence is the observed effect on $\alpha_{\text{IIb}}\beta_3$ phosphorylation, which supports integrin-based signaling and clot retraction.

How might the binding of ephrinB1 to its receptors support β_3 phosphorylation? It is likely that more than one mechanism is involved. The first mechanism reflects the ability of Eph/ephrin interactions to support the integrin engagement required for agonist-induced β_3 phosphorylation to occur. By contributing to aggregation, Eph/ephrin interactions increase the level of β_3 phosphorylation. In keeping with this first mechanism, we found that Eph/ephrin blockade inhibited ADP-induced platelet aggregation and ADP-induced β_3 phosphorylation. However, we also found that blockade of Eph/ephrin interactions had a greater effect on β_3 phosphorylation than on aggregation at low concentrations of ADP (Fig. 2) and that clustering ephrinB1 with GST-EphB1 was sufficient to cause β_3 phosphorylation. Therefore, we propose that there is a second mechanism by which Eph/ephrin interactions support β_3 phosphorylation, one that takes into account downstream signaling events in addition to Rap1 activation. Ephrin B family members are known to associate with Src family members and to

signal via cytoplasmic proteins bound to the ephrin PDZ target domain (4, 10). EphA4 becomes associated with two Src family members, Fyn and Lyn, during platelet activation (7). Therefore, the ephrin and its receptors can gather together kinases that can potentially phosphorylate β_3 , bringing those kinases into complexes that include the integrin. In keeping with such a mechanism, we found that β_3 phosphorylation in response to ephrinB1 clustering by GST-EphB1 is prevented by an inhibitor of Src family kinases. In contrast, Eph/ephrin interactions are clearly not the sole means by which Src family members are brought into contact with $\alpha_{IIb}\beta_3$. Arias-Salgado *et al.* (32) have recently reported that Src constitutively associates with β_3 integrins. Other Src family members (Fyn, Lyn, Hck, and Yes) were shown to associate with the cytoplasmic domain of β_3 when a fusion protein including that domain was incubated with platelet lysates. Might the association we observed previously between EphA4, Fyn, and Lyn be more simply attributed to the constitutive association of these kinases with $\alpha_{IIb}\beta_3$? Possibly, but this association seems unlikely to be the entire explanation. Although EphA4 is associated with $\alpha_{IIb}\beta_3$ in resting and activated platelets, we found previously that Fyn and Lyn associate with EphA4 only after the platelets are activated and that Src does not associate with EphA4 in either resting or activated platelets (7). Therefore, we propose that in addition to the constitutive association between $\alpha_{IIb}\beta_3$ and Src, there is an activation-dependent recruitment of other Src family members as a consequence of Eph/ephrin interactions, which, in turn, promotes the phosphorylation of β_3 . This contribution is particularly helpful at suboptimal agonist concentrations, but is needed less at high agonist concentrations.

The contribution of Eph kinases and ephrins to platelet function raises the larger issue of the role of contact-facilitated signaling in the later stages of platelet activation. There is increasing evidence that the boundaries between platelets within a growing thrombus are populated by adhesion and signaling molecules that help platelets remain in sustained contact with each other. Some of these molecules activate Tyr kinases. In addition to the Eph/ephrin interactions that are the subject of the present study, two laboratories have shown that platelets express receptors for Gas6, a vitamin K-dependent protein secreted from platelet α -granules (33, 34). Gas6 is a ligand for the Axl/Sky/Mer family of receptor Tyr kinases found on the surface of platelets. Like the Tyr \rightarrow Phe mutants of the β_3 tail, deletion of the genes encoding Gas6 or one of its receptors produces a phenotype of reversible platelet aggregation in mice. Deletion of these genes also causes resistance to

thrombosis (33, 35). It is reasonable to speculate that the signaling events downstream of the Axl receptor Tyr kinases will prove to have strong parallels with those downstream of Eph kinases in human platelets.

Once thrombus formation begins, contacts between platelets become increasingly stable and the spaces between platelets become increasingly constrained. Bioactive molecules that are secreted from platelet storage granules (such as ADP and Gas6), released from within the platelet (thromboxane A_2) or shed from the platelet surface (CD40L) can reach higher local concentrations at their receptors by being confined to a small space and protected from being washed out by blood flow. This contribution of clot retraction and, by extension, Eph/ephrin interactions may turn out to be one of the most relevant. Clot retraction would be expected to reduce the space between adjacent activated platelets, which increases the effective local concentration of bioactive molecules, a paradigm recently established for epithelial cells subjected to compressive stress (29). By promoting clot retraction, Eph/ephrin interactions may also limit the access of fibrinolytic enzymes to the clot interior and promote signaling through other cell surface receptors, including those that contribute to wound healing.

In conclusion, we have shown previously that in activated platelets Eph kinases and ephrins become part of a signaling complex that is not present in resting platelets. We now show that EphA4 is constitutively associated with $\alpha_{IIb}\beta_3$ and that Eph/ephrin interactions contribute to integrin outside-in signaling, helping to regulate clot retraction and improving aggregate stability. Although explored here in the context of platelets, we anticipate that the contribution of Eph kinases and ephrins to integrin outside-in signaling is not unique to platelets but will be found in other types of cells as well. Because they support thrombus growth and stability, Eph/ephrin interactions provide a novel target for the development of therapeutic antiplatelet agents that can be used in the prevention of strokes and recurrent coronary artery occlusions, adverse events that are ultimately due to the accumulation of inappropriate platelet aggregates. Although small-molecule inhibitors of Eph/ephrin interactions have not been described, a 12-residue peptide that selectively blocks ephrin binding to EphA4 was recently reported and may provide the basis for developing future therapeutic antagonists (36).

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