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Research Article **Hematology**

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Eptifibatide-induced thrombocytopenia and thrombosis in humans require FcγRIIa and the integrin β3 cytoplasmic domain

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Thrombocytopenia and thrombosis following treatment with the integrin αIIbβ3 antagonist eptifibatide are rare complications caused by patient antibodies specific for ligand-occupied αIIbβ3. Whether such antibodies induce platelet clearance by simple opsonization, by inducing mild platelet activation, or both is poorly understood. To gain insight into the mechanism by which eptifibatide-dependent antibodies initiate platelet clearance, we incubated normal human platelets with patient serum containing an αIIbβ3-specific, eptifibatide-dependent antibody. We observed that in the presence of eptifibatide, patient IgG induced platelet secretion and aggregation as well as tyrosine phosphorylation of the integrin β3 cytoplasmic domain, the platelet FcγRIIa Fc receptor, the protein-tyrosine kinase Syk, and phospholipase Cγ2. Each activation event was inhibited by preincubation of the platelets with Fab fragments of the FcγRIIa-specific mAb IV.3 or with the Src family kinase inhibitor PP2. Patient serum plus eptifibatide did not, however, activate platelets from a patient with a variant form of Glanzmann thrombasthenia that expressed normal levels of FcγRIIa and the αIIbβ3 complex but lacked most of the β3 cytoplasmic domain. Taken together, these data suggest a novel mechanism whereby eptifibatide-dependent antibodies engage the integrin β3 subunit such that FcγRIIa and its downstream signaling components become activated, resulting in thrombocytopenia and a predisposition to thrombosis.

Introduction

The integrin αIIbβ3 (also known as glycoprotein IIb-IIIa [GPIIb-IIIa]) is a member of the integrin family of cell adhesion receptors and is essential for normal hemostasis (1). Following platelet activation, the αIIbβ3 complex undergoes a dramatic conformational change that allows the adhesive protein fibrinogen to bind, forming a bridge between platelets that mediates platelet-platelet interactions and thrombus formation. Inappropriate activation of αIIbβ3 contributes substantially to cardiovascular disease (2) — a leading cause of death in the Western world (3). The development of effective fibrinogen receptor antagonists (FRAs), therefore, has been a major advance in the management of coronary artery diseases (4, 5).

Eptifibatide (Integrilin), one of several FDA-approved αIIbβ3 inhibitors, is a small, cyclic RGD-like heptapeptide that selectively inhibits ligand binding to the αIIbβ3 complex and rapidly dissociates from its receptor after cessation of therapy (6, 7). Eptifibatide has proven in numerous clinical trials to be effective in reducing the frequency of adverse outcomes in patients with acute coronary syndromes and secondary complications following percutaneous transluminal coronary angioplasty (8–11).

Despite their clinical efficacy, administration of all parenteral fibrinogen receptor antagonists, including eptifibatide, has been shown to increase the incidence of clinically significant thrombocytopenia (9, 10, 12–17). Though ligands that bind αIIbβ3 are capable of directly inducing both integrin and platelet activation (18–22), the acute thrombocytopenia that is infrequently observed after administration of eptifibatide is thought to be most commonly caused by the binding of either preexisting or neoantigen-induced drug-dependent antibodies (ddAbs) that bind to the αIIbβ3 complex in the presence of the drug (23). A recent case study suggests that thrombosis might also be an additional rare complication of eptifibatide therapy (24); however, whether this is antibody mediated has not been investigated.

Though the mechanism by which eptifibatide-dependent antibodies clear platelets from circulation has not been well examined, understanding the activating properties of other αIIbβ3-specific antibodies may provide relevant insights. For example, although the vast majority of murine mAbs that target the αIIbβ3 complex have no effect on platelet activation, several are potent stimulators. Anti-αIIbβ3-specific platelet-activating antibodies appear to fall into 2 broad categories. One class of mAbs, known as ligand-induced binding site (LIBS) antibodies, recognize conformational epitopes that are exposed upon integrin activation, ligand binding, or denaturation and activate platelets by stabilizing the open, or active, conformation of the integrin, enabling the binding of multivalent ligands such as fibrinogen (25–27). Antibody-mediated fibrinogen binding not only serves to bridge adjacent platelets but also initiates a broad series of reactions, collectively termed "outside-in" signaling, that augment a wide range of platelet activation responses, including shape change, granule secretion, and generation of cell-surface procoagulant activity (1).

The other class of activating αIIbβ3-specific murine mAbs all appear to bind in such a way as to present their Fc regions, either

Conflict of interest: P.J. Newman is a consultant for Novo Nordisk and a member of the Scientific Advisory Board of the New York Blood Center.

Nonstandard abbreviations used: ddAb, drug-dependent antibody; ITAM, immunoreceptor tyrosine-based activation motif; LIBS, ligand-induced binding site; SFK, Src family kinase.

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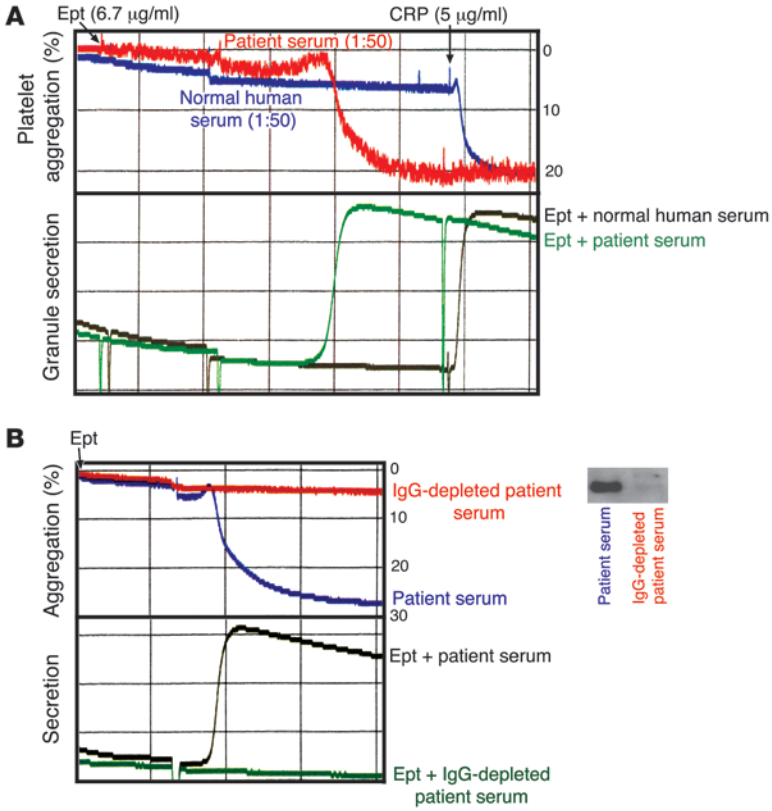

Figure 1
Serum IgG from a patient who developed severe thrombocytopenia and thrombosis following eptifibatide treatment induces platelet aggregation and granule secretion. (**A**) Washed human platelets were preincubated with eptifibatide (Ept) in a lumi-aggregometer for 3 minutes before addition of either patient or normal human serum. Collagen-related peptide (CRP) was added to the normal human platelets at the end of the experiment to demonstrate their ability to aggregate and secrete their granules. Note the scale on the *y* axis, indicating the blunted aggregation response in the presence of the drug. Granule secretion induced by patient antibodies, in contrast, was 100% of that induced by CRP, indicating the strong degree of platelet activation induced by the eptifibatide-dependent antibodies. (**B**) Patient serum was depleted of IgG using protein G Sepharose beads to reduce IgG content (SDS gel inset) and added to platelets in the presence of eptifibatide. Note that patient serum has lost its ability to activate platelets.

in *cis* (intraplatelet) or in *trans* (interplatelet) (28, 29) to the platelet Fc receptor FcγRIIa — a 40-kDa integral membrane protein (30) comprising 2 extracellular Ig-like domains, a single-pass transmembrane domain, and a 76-amino-acid cytoplasmic tail (31, 32) containing 2 YxxL sequences that together constitute a single immunoreceptor tyrosine-based activation motif (ITAM) (33, 34). Human platelets express, on average, approximately 3,000–5,000 copies of FcγRIIa per cell (35), and when the extracellular domains of these receptors become engaged or crosslinked, associated Src family kinases (SFKs) phosphorylate the ITAM tyrosines within the cytoplasmic domain (36), creating a docking site for the tandem Src homology 2 (SH2) domains of the protein-tyrosine kinase Syk (34, 37). Recruitment of Syk to the phosphorylated ITAMs at the inner face of the plasma membrane leads to its activation and subsequent assembly in lipid rafts of a multiprotein signaling complex consisting of the adaptor molecules Cbl (38) and LAT (39–41), the SFK Lyn (39, 42), PI3K (43–45), Tec family kinases Btk and Tec (46), and PLCγ2 (39, 45). Once activated, PLCγ2, via its lipase activity, generates lipid products that support a multitude of cellular activation responses, including integrin activation as well as platelet secretion and aggregation. Downregulation of FcγRIIa signaling appears to be accomplished through the activity of low-molecular-weight protein tyrosine phosphatase (LMW-PTP), which dephosphorylates the ITAM tyrosines of FcγRIIa (41). PECAM-1 has also been implicated in suppressing FcγRIIa function (47), though the mechanism by which this occurs has not yet been defined.

Despite accumulating evidence that thrombocytopenia associated with administration of fibrinogen receptor antagonists is immune in nature, the underlying mechanism by which this class of ddAbs cause platelet clearance and, less frequently, thrombosis, remains obscure. The purpose of the present investigation, therefore, was to gain further insight into etiology of eptifibatide-induced thrombocytopenia and thrombosis. Here, we provide the first evidence to our knowledge that these antibodies behave like the class II murine anti-platelet mAbs described above, in that their Fab regions interact with the αIIbβ3 complex, while the Fc region of the antibody activates FcγRIIa on the same cell. We also report the unexpected observation that the cytoplasmic domains of both integrin β3 and FcγRIIa are required for drug-induced antibody-mediated platelet activation to occur and propose what we believe to be a novel mechanism for thrombocytopenia and thrombosis following administration of fibrinogen receptor antagonists.

Results

An eptifibatide-dependent antibody that induces platelet secretion and aggregation. To gain further insight into the mechanism of eptifibatide-induced, antibody-mediated thrombocytopenia and thrombosis, we incubated normal human platelets with serum from a patient who had developed an eptifibatide-dependent antibody 2–3 days following administration of eptifibatide, and we simultaneously measured platelet aggregation and granule secretion in a lumi-aggregometer. As shown in Figure 1A, whereas normal human serum in the presence of eptifibatide had no effect on platelet activation, patient serum plus eptifibatide induced marked platelet aggregation that was blunted, as expected, due to the presence of the fibrinogen receptor antagonist eptifibatide. In contrast, the degree of granule secretion induced by patient serum plus eptifibatide approached that induced by strong agonists such as collagen or collagen-related peptide (CRP; black tracing, Figure 1A), demonstrating the potential for such antibodies to

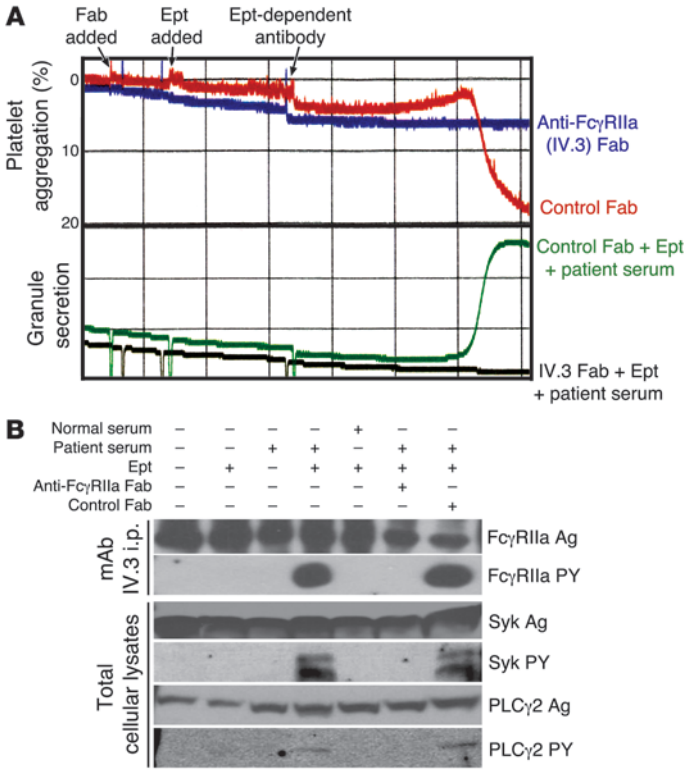

Figure 2
Requisite role for the FcγRIIa ITAM/Syk/PLCγ2 activation pathway in eptifibatide-dependent, antibody-induced platelet activation. (**A**) Fab fragments from normal mouse IgG or from the blocking anti-FcγRIIa mAb IV.3 were incubated with human platelets prior to addition of eptifibatide and patient serum. Note that blocking FcγRIIa with IV.3 Fabs totally abrogates both platelet aggregation and secretion induced by the patient IgG antibody. (**B**) Platelets undergoing eptifibatide-induced, antibody-dependent platelet aggregation in **A** were lysed directly in the aggregometer cuvette by adding 2× lysis buffer, as described in Methods. Levels of FcγRIIa, Syk, and PLCγ2 antigens (Ag) and of their tyrosine-phosphorylated counterparts (PY) were detected by Western blotting using antigen- or phosphotyrosine-specific antibodies, as indicated. Note that the tyrosine phosphorylation of all 3 signaling components induced by the binding of patient antibody is almost completely blocked by preincubation of platelets with IV.3, but not control, Fabs.

fully activate platelets. Platelet activation was found to be caused by antiplatelet antibodies in the patient serum, as removal of IgG using Protein G Sepharose beads completely abrogated the ability of the serum to activate platelets (Figure 1B). Addition of the ADP scavenger apyrase had no effect (data not shown), indicating that released ADP is not required for eptifibatide-dependent antibody-induced platelet aggregation.

Role of platelet Fc receptor signaling in eptifibatide-induced, antibody-mediated platelet activation. A small subset of murine mAbs specific for αIIbβ3 bind to platelets in such a way topographically as to present their Fc region to the platelet Fc receptor FcγRIIa and activate platelets (28, 29). Though there are as yet no accounts of human drug-dependent anti-αIIbβ3 antibodies that activate platelets via FcγRIIa, Pedicord et al. reported several years ago the production of a murine mAb that bound αIIbβ3 only in the presence of the oral αIIbβ3 antagonist roxifiban and activated platelets via interactions of its Fc region with FcγRIIa (48). To determine whether FcγRIIa might be similarly involved in platelet activation by human eptifibatide-dependent ddAbs, we repeated the experiment described in Figure 1, but with platelets that had been pretreated with Fab fragments of the FcγRIIa blocking antibody IV.3. As shown in Figure 2A, IV.3 Fabs completely blocked both granule secretion and aggregation induced by the eptifibatide-dependent antibody.

We obtained further evidence that FcγRIIa mediates platelet activation by the patient antibody by examining specific elements of the FcγRIIa signal transduction pathway (36, 37, 45). As shown in Figure 2B, addition of patient serum in the presence, but not absence, of eptifibatide resulted in phosphorylation of FcγRIIa ITAM tyrosines, the protein-tyrosine kinase Syk, and its downstream effector PLCγ2. These biochemical activation events were all blocked in platelets that had been preincubated with IV.3 Fabs, again demonstrating the requisite role for FcγRIIa in eptifibatide-dependent, antibody binding–induced platelet activation.

Phosphorylation of FcγRIIa ITAMs by Src family protein-tyrosine kinases is thought to be a early proximal event following engagement of the FcγRIIa extracellular domain (36), enabling the recruitment and activation of Syk to the inner face of the plasma membrane (34, 37, 43). To confirm that binding of eptifibatide-dependent patient antibodies activated the FcγRIIa/Syk/PLCγ2 pathway in an SFK-dependent manner, we pretreated platelets with the pan-Src inhibitor PP2 before adding eptifibatide and patient serum. As shown in Figure 3, PP2, but not its nonfunctional control analog PP3, completely blocked granule secretion, platelet aggregation, and signaling events downstream of FcγRIIa engagement induced by the eptifibatide-dependent antibody, confirming a requisite role for one or more SFKs in this process.

The integrin β3 cytoplasmic domain is required for ddAb-mediated platelet activation. As illustrated schematically in Figure 4A, eptifibatide-dependent antibodies engage the extracellular domains of the αIIbβ3 complexes via their Fab regions and FcγRIIa via their Fc regions. As a consequence, the potential exists for SFKs associated with the cytoplasmic domains of either the integrin β3 subunit (49–52) or FcγRIIa (39, 42) to function as ITAM kinases. To determine whether GPIIIa-associated SFKs might be required for initiating signaling downstream of antibody engagement, we examined the ability of eptifibatide-dependent antibodies to activate platelets that express normal levels (Figure 4B) of a truncated, mutant form of the integrin β3 subunit cytoplasmic domain that lacks SFK binding sequences (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI36745DS1). As was observed in a previously reported Glanzmann thrombasthenic patient harboring a Δ724 mutation (53),

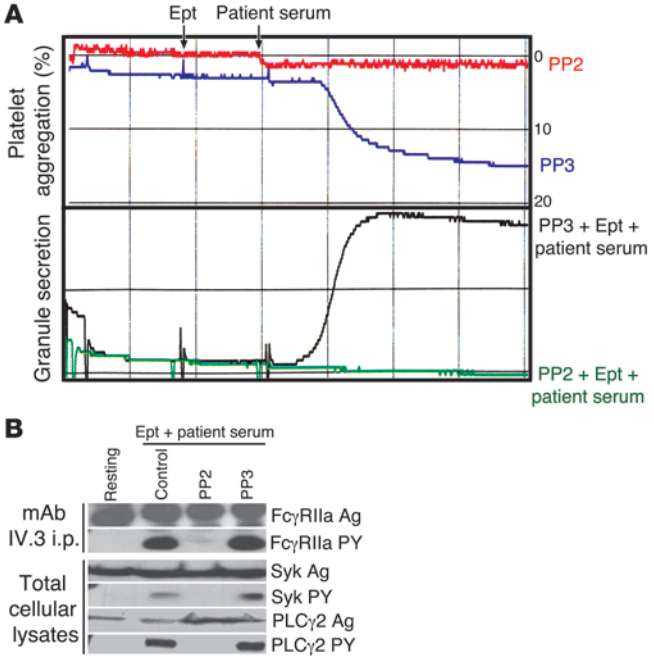

Figure 3
Role of SFKs in eptifibatide-dependent, antibody-induced platelet activation. Washed human platelets were pretreated with 25 μM pan-Src inhibitor PP2 or its nonreactive analog PP3 for 5 minutes at 37°C prior to addition of eptifibatide plus patient serum. (**A**) PP2 blocks antibody binding–induced platelet aggregation and secretion. (**B**) Dependency of SFKs on antibody binding–initiated activation of the primary components of the FcγRIIa signaling pathway.

the extracellular domain of this variant αIIbβ3 complex retains its normal structure and function, as evidenced by its ability to bind fibrinogen in the presence of Mn^{2+} (Supplemental Figure 1C) and bind the LIBS-specific mAb D3 (26) in an RGD-dependent manner (data not shown). FcγRIIa was also expressed at normal levels on these Glanzmann thrombasthenic platelets (Figure 4B) and able, upon engagement, to mediate robust granule secretion (Supplemental Figure 2) and platelet aggregation (data not shown), demonstrating that the FcγRIIa/SFK/Syk/PLCγ2 signal transduction pathway was fully operable in the platelets from this variant thrombasthenic patient. As shown in Figure 4C, however, despite having normal levels of αIIbβ3 on the surface and functional FcγRIIa-mediated signaling, platelets from the Δ724 variant thrombasthenic patient did not become activated by eptifibatide-dependent antibodies. These data demonstrate that functional FcγRIIa-associated SFKs in themselves are not sufficient to initiate eptifibatide-dependent, antibody binding–induced platelet activation. Rather, the αIIbβ3 cytoplasmic domain, and likely its associated SFKs, are also required.

Incidence of platelet-activating eptifibatide-dependent patient antibodies. Antibodies reactive with eptifibatide-coated platelets were detected, using a well-established, sensitive flow cytometric assay (23), in the serum of 26 of 42 patients referred to BloodCenter of Wisconsin's Diagnostic Platelet and Neutrophil Immunology Laboratory for suspected eptifibatide-induced thrombocytopenia (23). Serum from 3 of the 26 antibody-positive patients (12%) induced marked aggregation and granule secretion release of ATP from normal platelets treated with eptifibatide but had no effect on untreated platelets. Platelet activation induced by each of these 3 samples was completely blocked by mAb IV.3, suggesting a common mechanism of platelet activation — i.e., antibody-mediated bridging of the αIIbβ3 complex and FcγRIIa (data not shown).

Discussion

Though accumulating evidence supports an immune etiology for tirofiban- and eptifibatide-induced thrombocytopenia (23), exactly how tirofiban- or eptifibatide-dependent antibodies mediate platelet clearance is poorly understood. The observation that some tirofiban-dependent antibodies are capable of directly inducing platelet granule secretion (54, 55), together with a recent report of eptifibatide-induced thrombocytopenia associated with an increase in circulating procoagulant, platelet-derived microparticles (56), suggested to us that platelet activation might, in some instances, contribute to the occasional thrombocytopenia and, rarely, thrombosis (24) that are observed following administration of these αIIbβ3 ligand–mimetic compounds. The mechanism by which such antibodies might activate the platelets to which they are bound, however, is completely unknown.

The first antibody reported to activate human platelets as a consequence of its binding was a murine mAb specific for the tetraspanin CD9 (57). While most antiplatelet antibodies bind to the platelet surface without inducing platelet activation, a subset bind to their target antigens with a topographical orientation that causes them to elicit strong platelet activation, leading to granule secretion and platelet aggregation. The range of cell-surface receptors to which mAbs can bind and activate platelets is large and includes, in addition to CD9, the αIIbβ3 complex (58), CD36 (59), β2-microglobin (60), class I histocompatibility antigen (61), JAM-A (62), and the Gas6 receptors Axl, Sky, and Mer (63). With the exception of LIBS antibodies, which bind to or induce an active conformer of the αIIbβ3 complex (25–27), and antibodies to kinase domain–containing receptors (63), most of the remaining murine mAbs, including a recently described murine drug-dependent mAb specific for αIIbβ3 (48), appear to activate platelets by forming inter- or intraplatelet bridges between their target antigen and the platelet Fc receptor FcγRIIa (28, 29). Like their murine counterparts, human allo- (64), auto- (65, 66), and ddAbs induced by currently FDA-approved fibrinogen receptor antagonists (23, 54–56) have also been implicated in platelet activation. The mechanism by which such human antiplatelet antibodies activate platelets, however, is not known.

The major finding of the present work is that certain patient antibodies specific for the eptifibatide-bound αIIbβ3 complex activate platelets by engaging the integrin via their Fab regions and FcγRIIa via their Fc, regions. While there is no evidence for a direct physical association between αIIbβ3 and FcγRIIa, and they cannot be coimmunoprecipitated from detergent lysates (P.J. Newman and C. Gao, unpublished observations), they do appear to be topographically close to each other on the platelet surface, as evidenced by the finding that several αIIbβ3-specific mAbs, when prebound, are able to sterically block the binding of the anti-FcγRIIa mAb IV.3 (67, 68). Because αIIbβ3 complexes are present at relatively high density on the platelet surface (~40,000–80,000 per platelet; refs. 69, 70), it would seem that *any* αIIbβ3-bound antibody whose Fc domain is oriented in such a way as to engage a single FcγRIIa molecule, even though it is present at much lower density (3,000–5,000 copies/platelet; ref. 35), would have the potential to initiate FcγRIIa-mediated signaling. Further studies are needed to examine the range of antigen/drug/antibody

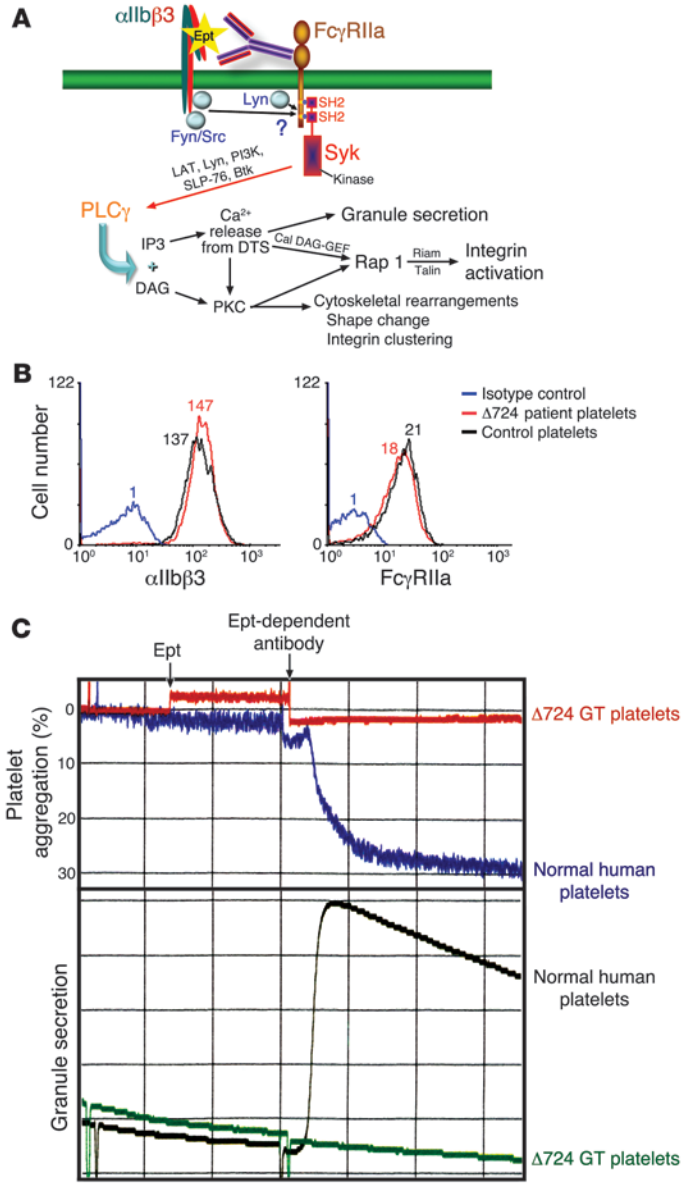

Figure 4
Evidence for β3 cytoplasmic domain–associated kinases in initiating eptifibatide antibody-induced platelet activation. (**A**) Schematic representation of an eptifibatide-dependent antibody simultaneously engaging both the αIIbβ3 complex and FcγRIIa, resulting in SFK-mediated phosphorylation of FcγRIIa ITAM tyrosines, recruitment of Syk, and activation of PLCγ2, ultimately resulting in platelet aggregation and granule secretion. Note that GPIIIa-associated Fyn and Src are brought into close proximity with FcγRIIa-associated Lyn as a result of antibody-mediated bridging of the extracellular domains of these 2 receptors. SH2, Src homology 2. Cal DAG-GEF is a guanine nucleotide exchange factor for Rap1. (**B**) Flow cytometric analysis of αIIbβ3 (detected with mAb AP2) and FcγRIIa (detected with mAb IV.3) expression on normal versus Δ724 Glanzmann thrombasthenic (GT) patient platelets analyzed in **C**. Note normal levels of both. Numbers above each peak indicate the median fluorescence intensity. Adapted with permission from the American Society of Hematology (82). (**C**) Failure of eptifibatide-dependent antibodies to activate FcγRIIa on platelets expressing a truncated β3 cytoplasmic domain.

combinations that can result in not only opsonization, but also activation, of platelets and thereby contribute to clinically relevant thrombocytopenia and occasional thrombosis.

Perhaps the most unanticipated finding of the present work is the strict requirement for the integrin β3 cytoplasmic domain in initiating platelet activation induced by eptifibatide-dependent patient antibodies. Though the molecular components are different, the mechanism of action by which such antibodies are able to induce platelet secretion and aggregation is not unlike that underlying cytokine and growth factor receptor signaling, in which homo- or heterodimeric receptors are brought into close approximation, resulting in transactivation of intrinsic or associated tyrosine kinases. Similarly, when the extracellular domains of 2 or more FcγRIIa molecules are brought together by IgG immune complexes (often simulated experimentally by addition of heat-aggregated IgG or mAb IV.3 plus anti-mouse IgG), homodimerization or multimerization occurs, allowing an SFK-mediated chain reaction to begin (illustrated in Supplemental Figure 2), quickly resulting in robust platelet activation. Likewise, eptifibatide-dependent antibody-mediated clustering of αIIbβ3, via the Fab domain of the antibody, with FcγRIIa, via its Fc region (illustrated schematically in Figure 4A), leads to transactivation of integrin- and Fc receptor–associated protein tyrosine kinases, which function either directly or indirectly as ITAM kinases to facilitate the assembly of a signaling complex that initiates platelet activation. Evidence for this model derives from the observation that addition of the SFK inhibitor PP2 completely abrogates platelet activation by eptifibatide-dependent antibodies (Figure 3) and that such patient antibodies are unable to activate platelets that express a mutant integrin lacking most of the cytoplasmic domain of GPIIIa (Figure 4). Whether other molecular players are involved in eptifibatide-dependent antibody-induced platelet activation, and whether all activating patient antibodies act via the same mechanism, is currently under investigation.

The observation that a human eptifibatide-dependent antibody can initiate FcγRIIa-mediated signal transduction leading to granule secretion and residual platelet aggregation in the presence of the potent αIIbβ3 antagonist eptifibatide (Figures 1–4) strongly suggests that αIIbβ3-independent events are involved. McGregor et al. showed nearly 20 years ago that platelets from a patient with type I Glanzmann thrombasthenic exhibited residual aggregation and near-normal granule secretion in response to stimulation with collagen (71) — a strong agonist that activates platelets via essentially the same ITAM/Syk/PLCγ2 pathway employed by FcγRIIa. In experiments not shown, eptifibatide-dependent, antibody-mediated platelet aggregation was induced in the presence of a 10-fold-higher concentration of eptifibatide than that employed in Figures 1–4 (i.e., 67.0 versus 6.7 μg/ml) or in the presence of patient serum plus eptifibatide plus 20 mg/ml of AP2 — an αIIbβ3 complex–specific mAb that blocks both fibrinogen binding and platelet aggregation (72). Taken together, these data support the notion that patient antibodies bridging αIIbβ3 and FcγRIIa induce platelet granule secretion and residual aggregation in a αIIbβ3-independent manner. This αIIbβ3-independent pathway of aggregation may be restricted to circumstances where αIIbβ3 blockade occurs or may even be activated under such circumstances. It is also possible that αIIbβ3-independent

aggregation observed here may be restricted to specific individuals. Because RGD-containing ligands such as fibrinogen, vWF, and fibronectin are prevented from binding to αIIbβ3 in the presence of eptifibatide, other receptor/ligand pairs are likely to be mediating platelet-platelet interactions. While we have not yet addressed this issue, CD36/thrombospondin, P-selectin/PSGL-1, and GPIb/vWF all seem like plausible candidates, since ligands for each of these receptor/ligand pairs are released from platelet α-granules following FcγRIIa-mediated platelet activation.

Finally, given (a) that the density of FcγRIIa can vary by as much as 2- to 3-fold from individual to individual (35) and that the level of FcγRIIa expression likely affects platelet responsiveness (73–76); (b) that the 2 allelic isoforms (Arg131 versus His131) of FcγRIIa might also contribute to its ability to stimulate platelets (74, 77); and, as reported herein, (c) the observations of the obligatory involvement of FcγRIIa in thrombocytopenia and thrombosis in a large subset of eptifibatide-dependent patient antibodies, further studies appear to be needed to examine whether prescreening patients for FcγRIIa genotype and/or expression level are warranted before administration of αIIbβ3 antagonists.

Methods

Patient studies. The index case — a 73-year-old man admitted to a local hospital with partial obstruction of the right coronary artery and a platelet count of 212,000/μl — was one of 42 patients referred to the BloodCenter of Wisconsin's Diagnostic Platelet and Neutrophil Immunology Laboratory who developed thrombocytopenia after being given eptifibatide for prevention of thrombotic complications following percutaneous transluminal coronary angioplasty (23). The patient was given intravenous infusions of unfractionated porcine heparin and eptifibatide prior to stent implantation. Approximately 12 hours later, he suffered an acute inferior wall myocardial infarction. A thrombosed stent in the right coronary artery was reopened and eptifibatide was restarted with heparin and clopidogrel. Eighteen hours later, a large hematoma was identified at the site of catheter entry in the right groin, and his platelet count dropped to 25,000/μl. Heparin and eptifibatide were discontinued. Serological analysis for heparin-dependent antibodies was negative, but eptifibatide-dependent antibodies were found by flow cytometry. Platelet counts remained less than 38,000/μl for the next 3 days. On day 6, a deep venous thrombosis was identified in the right leg and treated with warfarin and low-molecular-weight heparin. The platelet count rose to 244,000/μl on day 7, and the patient was discharged on day 9 with no further hematologic or cardiac abnormalities for the next 6 months.

Reagents and antibodies. The synthetic peptides RGDW and CRP (78) were synthesized by the Protein Chemistry Core Laboratory at the Blood Research Institute, BloodCenter of Wisconsin. Eptifibatide was obtained by prescription from a local pharmacy. Luciferase and PGE_1 were purchased from Chrono-Log Corp. and Sigma-Aldrich, respectively. Wortmannin, PP2, and PP3 were from Calbiochem. The αIIbβ3-specific mAbs AP2 and AP3 have been previously described (69, 79). The hybridoma cell line secreting the FcγRIIa-specific mAb IV.3 (30, 80) was purchased from ATCC. A nonblocking FcγRIIa-specific mAb conjugated to FITC was obtained from BD Biosciences — Pharmingen. Polyclonal antibodies specific for PLCγ2, phosphotyrosine$_{759}$ of PLCγ2, and phosphotyrosine$_{525,526}$ of Syk were purchased from Cell Signaling Technology. Mouse anti–human Syk and normal human IgG were obtained from Santa Cruz Biotechnology Inc. Anti–phospho-tyrosine mAb (PY-20) was from Zymed. AP2 and AP3 were labeled with Cy3 by using a commercial kit from GE Healthcare, while mAb IV.3 was labeled with Alexa Fluor 488 by using a labeling kit from Invitrogen. Fab fragments of mAbs IV.3, AP2, and AP3 were produced by using IgG1 Fab and $F(ab')_2$ Preparation Kit (Pierce Biotechnology; Thermo Scientific). Purified fibrinogen was provided by Michael Mosesson (Blood Research Institute, BloodCenter of Wisconsin) and labeled with FITC according to previously described methods (81).

Platelet aggregation and secretion. All studies using human patient samples were reviewed and approved by the Institutional Review Board of the BloodCenter of Wisconsin, with appropriate informed consent of the participants. Blood samples were collected into 3.8% sodium citrate, diluted 1:1 with modified Tyrode's-HEPES buffer, and then allowed to "rest" by incubation at room temperature for 10 minutes. Platelet-rich plasma (PRP) was prepared by low-speed centrifugation, washed into modified Tyrode's-HEPES containing 50 ng/ml PGE_1 and 5 mM EDTA, and finally resuspended in Tyrode's-HEPES containing 1 mM $CaCl_2$ to a final concentration of 3.0×10^8/ml. Platelet aggregation was performed at 37°C in a Chrono-Log whole blood lumi-aggregometer in the presence of luciferase to simultaneously measure light transmission and secretion of dense granule–derived ATP. For selected studies, platelets were obtained, with parent-provided informed consent, from a two-year-old male child with a variant form of Glanzmann thrombasthenia. DNA sequence analysis revealed a C2268T homozygous mutation within exon 13 of the patient's β3 gene that encodes an Arg724Stop mutation in both alleles (Supplemental Figure 1). This results in the expression of a truncated form of GPIIIa whose cytoplasmic domain contains only 8 of 47 residues. The expression levels of this mutant αIIbβ3 complex on the platelet surface were normal (see below).

Immunoprecipitation and Western blot analysis. Platelet detergent lysates were prepared by adding and equal volume of ice-cold 2× lysis buffer (30 mM HEPES [pH 7.4], 300 mM NaCl, 20 mM EGTA, 0.2 mM $MgCl_2$, 2% Triton X-100) containing 2× protease and phosphatase inhibitor cocktail (Calbiochem; EMD) directly to the aggregometer cuvette. Syk, phospho-Syk, PLCγ2, and phospho-PLCγ2 were examined by Western blot analysis of total platelet lysates, while the phosphorylation state of FcγRIIa was measured after immunoprecipitation with IV.3, followed by capture of immune complexes using protein G Sepharose beads (Amersham Biosciences; GE Healthcare). Following SDS-PAGE, immunoprecipitated proteins were transferred to polyvinylidene fluoride membranes and visualized using an ECL detection kit (Amersham Biosciences; GE Healthcare).

Flow cytometry. Washed human platelets (50 μl) at a concentration of 2×10^8/ml were incubated with 5 μg/ml of the indicated mAbs for 1 hour at room temperature. Platelets were washed in 1 ml HEN (0.1 M HEPES, 1 mM EDTA, 50 mM NaCl, pH 7.4), resuspended in 2 μg/ml PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) for 45 minutes, and then analyzed on a BD LSR II flow cytometer. In some experiments, the platelet activation state was evaluated by the addition of 125 μg/ml of FITC-labeled fibrinogen in the presence of 1 mM Mn^{2+} versus 2 mM EDTA.

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