Disabled-2 regulates platelet heterotypic and homotypic aggregation through sulfatide binding

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
in
Biological Sciences

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March 29th, 2010
Blacksburg, Virginia

Keywords: Disabled-2, sulfatides, platelets, leukocytes

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Abstract

At the site of vascular injury platelet aggregation serves to stem blood flow, initiate the inflammatory response, and stimulate wound healing. Platelets become stimulated, release their granule contents, and become adherent to one another. Platelet granules contain important clotting factors and regulators of aggregation. Disabled-2 (Dab2) is a negative regulator of platelet aggregation released from platelet α-granules. Dab2 binds to the $\alpha_{IIb}\beta_3$ integrin, through the PTB domain, and blocks fibrin binding to the integrin which serves as the major cause of platelet-platelet interactions. Dab2 is also capable of binding to sulfatides, through the N-PTB region, expressed on the outer leaflet of adjacent cells. Dab2-sulfatide binding decreases Dab2's ability to interact with the $\alpha_{IIb}\beta_3$ integrin, however sulfatides activate and stimulate platelet-platelet and platelet-leukocyte aggregation. Sulfatide addition to platelets stimulates increased $\alpha_{IIb}\beta_3$ integrin and P-selectin expression through stimulation of continued platelet degranulation, and these surface receptors mediate platelet heterotypic and homotypic aggregation.

Here, we show that Dab2 N-PTB binding of sulfatides serves to increase the inhibitory affect of Dab2. Sulfatide stimulation of platelet degranulation can be blocked by the addition of N-PTB. Inhibition of sulfatide induced $\alpha_{IIb}\beta_3$ integrin and P-selectin expression result in decreased platelet-platelet aggregation under flow. N-PTB also blocks sulfatide induced platelet-leukocyte interactions and aggregation. Experimental data supports the hypothesis that Dab2-sulfatide binding serves to increase the inhibition of platelet aggregation.

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Dedication

To my family, whose love, support, and sarcastic teasing has been a source of constant motivation and comfort.

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Chapter 1: Background and Significance

1. Platelet Function

Platelets (or thrombocytes) are cell fragments that circulate in the blood stream, and become stimulated and form aggregates at the site of vascular injury [1]. Platelets interact with one another via fibrinogen/fibrin linking adjacent platelets together to form a haemostatic plug [2]. Platelets are a natural source of growth factors and cytokines, which stimulate tissue growth, wound healing, and inflammatory cell recruitment at the site of vascular injury [7,8].

1.1 Platelet activation and aggregation

Endothelial cells, which line blood vessels, are separated from the basement membrane via a layer of secreted von Willebrand factor (vWF). When endothelial cells are damaged the vWF layer is exposed to the flowing blood [10]. Circulating in the blood are clotting factors including Factor VIII and collagen which are recruited to the vWF. The collagen provides a site for platelet adhesion by binding to the platelet glycoprotein Ia/IIa (GPIa/IIa) surface receptors [13]. Stabilized vWF also tethers platelets through surface glycoprotein Ib-IX-V (GPIb-IX-V) complex binding [13]. The binding of collagen to the GPIa/IIa and vWF to GPIb-IX-V receptors results in platelet activation and release of the platelets α- and δ-granule contents [11, 13]. The granules contain adhesive proteins, coagulation

factors, protease inhibitors, as well as activating factors ADP, serotonin, platelet activating factor (PAF), and thromboxane A_2 (TXA_2) [9, 14]. Once released the granule contents interact with other circulating platelets and trigger their activation through a G-linked protein receptor cascade [9]. The activation of the G-linked protein receptor cascade leads to increasing concentration of cytosolic calcium, activation of protein kinase C and phospholipase $C\gamma_2$ ($PLC\gamma_2$). These events converge in the activation of the $\alpha_{IIb}\beta_3$ integrin receptor, through a conformational change, which is responsible for binding to fibrinogen and fibrin linking platelets together in the growing aggregate [9, 10, 11, 21].

1.2 Platelet function in inflammation

Platelets have been shown to contribute to the inflammatory response during their activation, adhesion, and aggregation [3]. Endothelial bound platelets provide a surface for leukocyte rolling, adhesion, and transmigration into surrounding tissue [4]. In the blood stream activated platelets mediate microemboli formation containing leukocytes which increase adhesion and tissue accumulation [5-6]. The surface expression of P-selectin on activated platelets mediates platelet-leukocyte interactions and increases leukocyte recruitment [24, 25]. During thrombus growth activated platelets release proinflammatory cytokines which serve to activate leukocyte integrins and mediate their accumulation [7]. Platelet factor 4 which is

released from activated platelet α-granules is chemotactic for neutrophils, fibroblasts, and monocytes [28]. Platelet activating factor also mediates many leukocyte functions at the site of platelet aggregation [26, 27]. Platelet recruitment and activation of circulating leukocytes are key for proper immune response at the site of vascular injury [3].

1.3 Platelet function in disease

Myocardial infarction and strokes are often the result of arterial thrombosis. The buildup of arterial lipid plaques result in narrowing of the artieries, and upon plaque ruptures rapid thrombus formation which can block proper blood flow [13]. Increased levels of platelet-leukocyte microemboli are pro-inflammatory and increase the risk of formation of arterial thrombi. In patients with both coronary angioplasty and unstable angina increased levels of platelet-leukocyte aggregates were observed [29, 30]. Increased levels of platelet leukocyte aggregates facilitate leukocyte recruitment to the sites of vascular injury. Leukocyte invasion to the surrounding tissue leads to hardening of the tissue and progression of atherosclerotic lesions [29, 30]. Mediating platelet function in both thrombus formation and induction of inflammation has been a therapeutic target for prevention of atherosclerosis, myocardial infarction, and stroke [12].

2. Disabled-2 (Dab2)

Disabled-2 (Dab2) is a modular protein that acts as an adaptor in endocytosis and canonical Wingless Type (Wnt) signaling [15, 16]. Dab2 is contained within the α-granules of resting platelets and is released upon activation [17]. When released Dab2 has been identified as a negative regulator of platelet aggregation through $\alpha_{IIb}\beta_3$ integrin binding [17]. Dab2 also binds to lipids, including sulfatides [19, 18]. The integrin and lipid binding sites overlap in the N-terminal Phosphotyrosine Binding/Interaction Domain (PTB) resulting in the establishment of multiple pools of Dab2 located at the platelet surface based on their respective binding partner [16, 18].

2.1 Dab2 Domain Structure

Dab2 contains two distinct functional domains; a C-terminal proline-rich domain (PRD), and an N-terminal PTB domain, a member of the Dab Homology domain family (Figure 1.1a) [19, 20]. The PTB domain (33-241) is capable of binding to both peptides and lipids in two separate pockets based on structural analysis of the homologous Dab1 PTB domain [19]. Inhibition of the $\alpha IIb\beta_3$ integrin by Dab2 is mediated by an RGD motif (amino acids 64-66) on the PTB domain which interacts with the αIIb-integrin–fibrinogen binding region (amino acid residues 171-464) [17]. At the residue 34 there is a thrombin cleavage site that results in the degradation and inactivity of Dab2 as an integrin inhibitor [17,

18]. The PTB domain contains three of four residues required for sulfatide binding, K49, K51, and K53, with the final residue needed for sulfatide binding is located in the N-terminal region of Dab2, K25 (Figure 1.1a) [18]. The binding sites for the integrin and sulfatides are close proximity in the 3D structure of mouse Dab2 PTB domain (Figure 1.1b) [19]. While Dab2 is capable of binding to peptide and lipid ligands simultaneously, the binding of Dab2 to either platelet integrin or sulfatides during platelet aggregation does not happen simultaneously [18, 19].

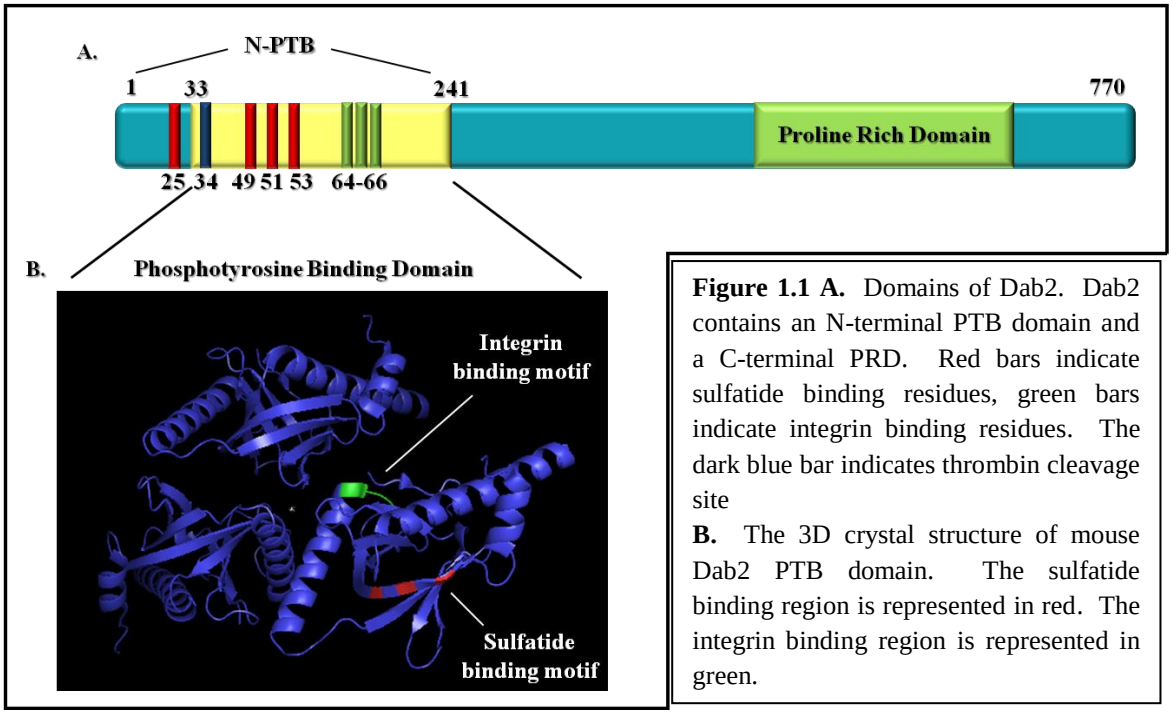


Figure 1.1 A. Domains of Dab2. Dab2 contains an N-terminal PTB domain and a C-terminal PRD. Red bars indicate sulfatide binding residues, green bars indicate integrin binding residues. The dark blue bar indicates thrombin cleavage site

B. The 3D crystal structure of mouse Dab2 PTB domain. The sulfatide binding region is represented in red. The integrin binding region is represented in green.

2.2 Dab2 binding partners

Dab2 binds to a number of protein and lipid partners including, α_{IIb}-integrin, clatherin, Dishevelled-3, Axin, PtdIns(4,5)P_2, and sulfatides all through its PTB domain [17, 31, 19, 18] . During platelet aggregation events Dab2 is released extracellularly and recruited to either the $\alpha_{IIb}\beta_3$ integrin or to surface sulfatides creating two pools of Dab2 [18].

2.2i. $\alpha_{IIb}\beta_3$ integrin

The $\alpha_{IIb}\beta_3$ integrin is the most abundant surface integrin receptor on a platelet. A resting platelet contains roughly 80,000 receptors [21]. In a resting state the receptor has an inactive conformation that blocks the fibrinogen-binding region, which recognizes Arg-Gly-Asp (RGD) residues [21]. Upon platelet activation outside in G-protein coupled signaling events lead to a conformational change exposing the fibrinogen-binding region [23]. The $\alpha_{IIb}\beta_3$ integrin recognizes two RGD motifs in the α chain of fibrinogen, RGDF (residues 95-98) and RGDS (residues 572-575) [17]. The binding of fibrinogen cross-links platelets in the growing aggregate and triggers outside-in signaling events through Src and Syk tyrosine kinases to regulate PLCγ function resulting in changes in lamellipodia formation [22]. The $\alpha_{IIb}\beta_3$ integrin eventually causes shifts in lipid raft locations

that recruits actin-interacting proteins to the cytoskeleton which triggers clot retraction [23].

Dab2 is able to prevent fibrinogen from interacting with the $\alpha_{IIb}\beta_3$ integrin both intracellularly and extracellularly. Cytosolic Dab2 can be phosphorylated on its serine 24 residue, and then interact with the ctyosolic tail of the β_3 subunit which renders the integrin inactive (Figure 1.2) [17]. Extracellularly Dab2 is able to compete with fibrinogen for α_{IIb} binding upon its release from platelet α-granules through its RGD binding motif (Figure 1.2). Platelet aggregometer studies have shown a decreased aggregation in the presence of recombinant PTB [17]. Platelet-fibrinogen adhesion assays as well as flow cytometry assays have both demonstrated binding between Dab2 PTB and the $\alpha_{IIb}\beta_3$ integrin. When residue D66 was mutated to E (N-PTB^{D66E}), to disrupt the RGD motif, integrin binding was eliminated further suggesting that this motif was responsible for the direct association [17, 18].

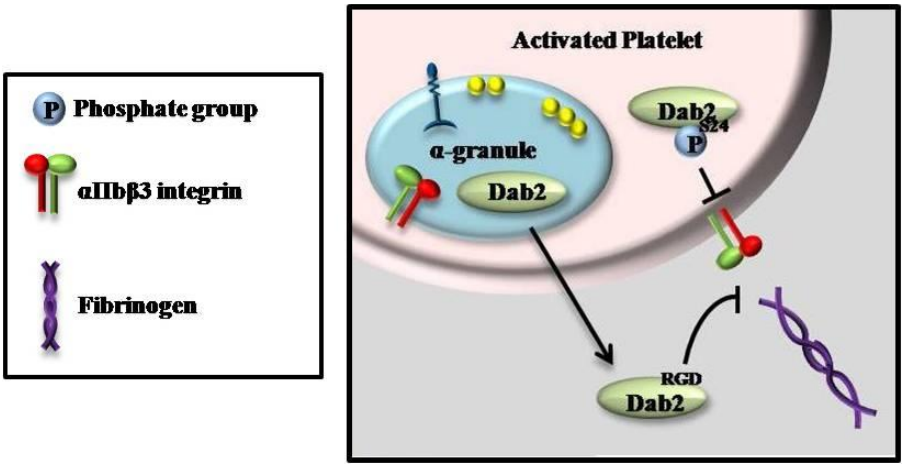
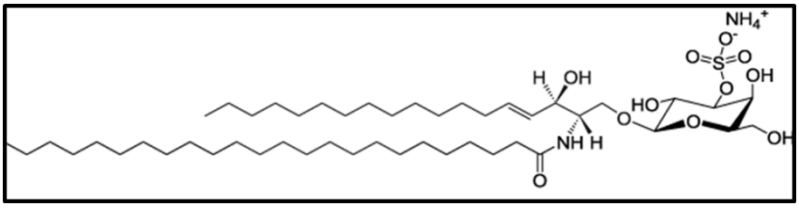


Figure 1.2. Dab2 inhibition of fibrinogen binding to $\alpha_{IIb}\beta_3$ integrin. Intracellularly Dab2 phosphorylated on S24 inactivates the $\alpha_{IIb}\beta_3$ integrin through the β_3 cytosolic tail. Extracellularly Dab2 is released from α-granules and binds to the α_{IIb} subunit via its RGD motif blocking fibrinogen.

2.2ii. Sulfatides

Sulfatides are sulfated galactosylceramides which are synthesized by the enzyme cerebroside sulfotransferase [46]. Sulfatides contain a ceramide backbone with a sulfate head group and two fatty acid chain tails which contain between 18-24 carbons [42] (Figure 1.3). Sulfatides are found mostly on the outer membrane in neuronal cells, glandular epithelial cells, platelets, erythrocytes, granulocytes, and certain cancer cells [32, 33, 34, 35]. Sulfatides function in cell adhesion and nerve conduction [36, 37].

Figure 1.3 Chemical Structure of the predominant sulfatide species, and Dab2 ligand.



Sulfatides are capable of binding to several important adhesion proteins in the coagulation process including thrombospodin, vWF, laminin, and selectins [35, 38, 39, 40]. When platelet surface sulfatides interact with P-selectin of adjacent platelets it stabilizes clot formation [34]. P-selectin binding to sulfatides also triggers an internal pathway thought to be mediated by p38 that results in increased α-granule release increasing surface $\alpha_{IIb}\beta_3$ integrin and P-selectin [24]. Increased degranulation augments the platelets ability to interact with other platelets through its $\alpha_{IIb}\beta_3$ integrin binding to fibrinogen as well as P-selectin binding directly to the

sulfatides of adjacent platelets. Sulfatides, induce P-selectin expression which increases the ability of platelets to interact with circulating leukocytes through a P-selectin dependent interaction with leukocyte P-selectin glycoprotein ligand-1 (PSGL-1), as well as directly activating leukocytes through L-selectin dependent and independent pathways [24, 41].

Our previous studies showed Dab2 binding to sulfatides is mediated through the PTB domain residues K25, K49, K51, and K53 [18]. When the N-terminal-PTB portion of Dab2 (N-PTB) is exposed to sulfatide enriched liposomes these residues each individually contributes to the binding, and mutation of all four residues from positively charge lysines to alanines ($N\text{-}PTB^{4M}$) lead to an abolition of sulfatide binding. The binding kinetics (K_D) of wild type N-PTB is K_D= 0.6 μM. When N-PTB is bound to sulfatides the internal thrombin cleavage site becomes inaccessible affording the protein cleavage protection [18]. Abolition of sulfatide binding increased the ability of N-PTB to bind to the $\alpha_{IIb}\beta_3$ integrin by increasing the amount of free N-PTB, and in both platelet adhesion and flow cytometry assays it was shown that $N\text{-}PTB^{D66E}$ is incapable of binding to the $\alpha_{IIb}\beta_3$ integrin, N-PTB is able to inhibit the binding and this inhibition is increased with the abolition of sulfatide binding using $N\text{-}PTB^{4M}$ (Figure 1.4) [18].

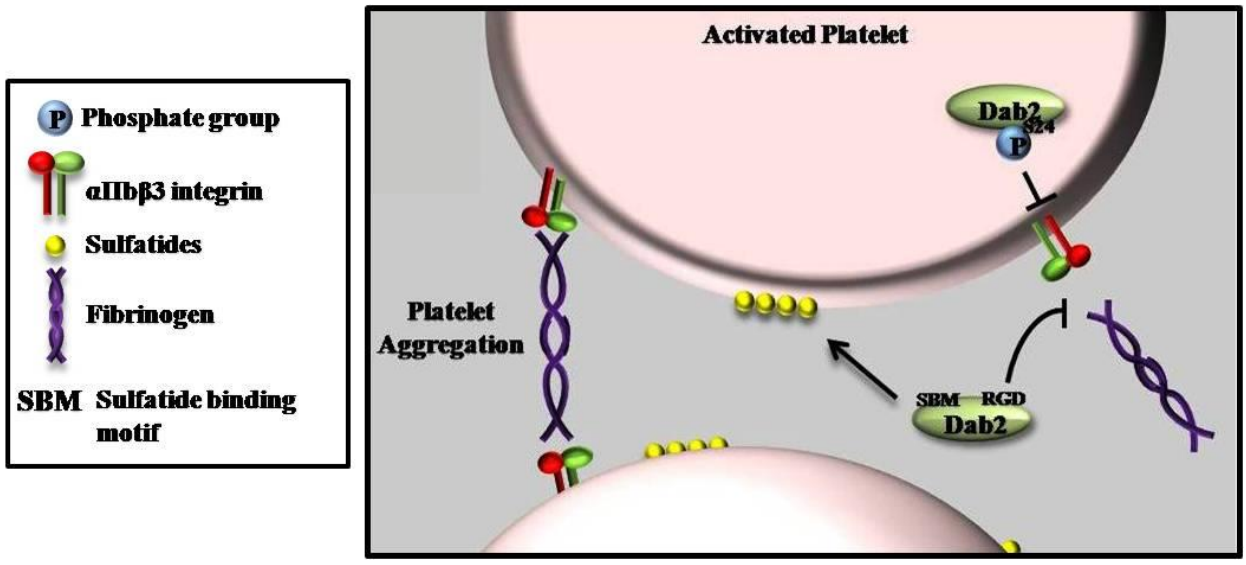


Figure 1.4 Two pools of Dab2 exist on the platelet surface. One pool binds to the $\alpha_{IIb}\beta_3$ integrin and blocks fibrinogen binding. The second pool interacts with surface sulfatides and does not prevent fibrinogen binding.

Localization of N-PTB to the surface of platelets through sulfatide binding also increased the amount of N-PTB internalized during platelet activation progression. Endogenous Dab2 becomes internalized 10 minutes after Thrombin Receptor Activating Peptide (TRAP) addition to platelets. Sulfatide binding resulted in an increase of membrane localized N-PTB when added exogenously and in turn this resulted in an increase in internalized N-PTB. The role of internal Dab2 post activation is unknown but sulfatide binding is essential for any possible recycling of Dab2 [18].

Chapter 2: Specific Aims

The overall goal of our research is to elucidate the role of Dab2 binding of sulfatides during platelet heterotypic and homotypic aggregation. Through the generation of mutant Dab2 N-PTB constructs we are able to control the localization of N-PTB to specific binding partners and monitor the effects on platelet activity. Using platelet aggregation assays to determine how N-PTB mediated changes in activity related to platelet function in both platelet-platelet and platelet-leukocyte interactions.

Based on our preliminary data, we hypothesize that Dab2 binding of sulfatides contributes to its overall function as an inhibitor of platelet aggregation, and that this interaction may affect clot stability, platelet activation, and platelet aggregation. Does sulfatide localization of Dab2, away from the $\alpha_{IIb}\beta_3$ integrin, decrease or complement Dab2's function as an inhibitor of platelet aggregation? In the presence of sulfatides does Dab2 compete with platelet P-selectin for binding, and how does this affect platelet function? Is Dab2 able to affect platelet-leukocyte interactions through sulfatide binding? To address these questions we have applied flow cytometry analysis, intercellular binding assays, and platelet aggregation and functional assays under flow conditions as presented within the following aims:

Aim I. To determine how sulfatide binding affects Dab2's role as an inhibitor of platelet aggregation.

Dab2 is released from platelet α-granules upon platelet activation and once released it localizes to the platelet surface to either, the $\alpha_{IIb}\beta_3$ integrin through its PTB RGD motif, or to platelet surface sulfatides through its N-PTB sulfatide binding motifs. Sulfatides function in platelet aggregation to stabilize clot formation as well as to increase platelet degranulation through binding with platelet P-selectin [24, 34]. Increased platelet degranulation augment surface $\alpha_{IIb}\beta_3$ integrin, P-selectin, and platelet aggregation [24]. We hypothesize Dab2 inhibits platelet activation and aggregation though a dual mechanism: through $\alpha_{IIb}\beta_3$ integrin binding, and through inhibition of sulfatide induced platelet activation.

To monitor the ability of sufatides to induce platelet activation we will study degranulation markers on the platelets surface, P-selectin (CD62P) and the $\alpha_{IIb}\beta_3$ integrin. Surface expression of the markers will be evaluated in the presence of sulfatide liposomes, ADP stimulated platelets, and N-PTB. Mutant forms of N-PTB will also be used to determine how binding partners affect platelet activation. In order to determine the functional affect of sulfatide stimulated degranulation we have established a clot formation under flow assay to monitor the rate of platelet thrombus growth over time.

Aim II. Determine how Dab2 mediates platelet recruitment of leukocytes in the presence of sulfatides.

P-selectin can bind to PSGL-1 or surface sulfatides expressed by leukocytes, and these microemboli can then be incorporated into growing clots [24]. Thus we hypothesize that Dab2 inhibits platelet driven leukocyte recruitment: by inhibiting sulfatide induced platelet expression of P-selectin and leukocyte activation.

To examine platelet-leukocyte interactions in the presence of sulfatides we were able to monitor platelet-leukocyte aggregates. To monitor the inhibitory affects of N-PTB we will monitor leukocyte bound platelets using flow cytometry. To examine leukocyte adhesion under flow conditions we utilized the plasma protein coated flow chamber assay and monitored platelet-leukocyte aggregate formation.

Chapter 3: Results

P-selectin is a transmembrane glycoprotein, contained in the α-granules of platelets [43]. P-selectin is transported to the platelet surface upon the release of the α-granules during agonist induced platelet activation [45]. Once P-selectin is expressed on the platelet surface it mediates platelet adhesion to activated platelets, monocytes, and neutrophils. P-selectin mediates platelet adhesion to monocytes and neutrophils via binding to P-selectin glycoprotein ligand 1 (PSGL-1) [5]. However, platelet-platelet interactions are mediated by P-selectin binding to surface sulfatides, which come to the platelet's surface after activation, of adjacent platelets. This binding stabilizes platelet-platelet aggregates during clot formation [24]. P-selectin binding of sulfatides also induces further degranulation of the platelet, which leads to increased levels of surface P-selectin [34]. An increase in surface P-selectin increases platelet aggregation as well as platelet binding of leukocytes [34]. The P-selectin expressed on the surface of endothelial adherent platelets provide a binding site as well as proinflammatory compounds (IL-1β, CD40L) for leukocytes to bind and become inflamed. It is believed that the inflamed leukocytes may then migrate from the blood stream into the surrounding tissue causing tissue damage and plaque formation, as elevated levels of circulating activated platelets have been linked to atherosclerosis in humans [12].

Dab2 is a modular protein contained in the α-granules of platelets, and is released upon platelet activation and degranulation [17]. Once released Dab2 interacts with the extracellular portion of the $\alpha_{IIb}\beta_3$ integrin on the platelet surface via an RGD motif contained in the phospho-tyrosine binding (PTB) domain [44]. Dab2 competes with fibrinogen for $\alpha_{IIb}\beta_3$ integrin binding; this competition inhibits clot formation [44]. Previously we have shown Dab2 binds to sulfatides via two motifs located between the N terminus and the PTB domain (N-PTB) of Dab2 [18]. Dab2 binds to platelet surface sulfatides with high affinity, and this interaction mediates the location of Dab2, either integrin bound or sulfatide bound. Here, we investigate Dab2 binding of platelet surface sulfatides and how this affects Dab2 function. We found that Dab2 binding of sulfatides prevents further activation of platelets leading to decreased amounts of surface P-selectin and in turn platelet heterotypic and homotypic aggregation.

3.1 Sulfatide binding to P-selectin triggers degranulation of ADP stimulated platelets.

Resting platelets do not express P-selectin on their surface, however after stimulation with an agonist and subsequent degranulation P-selectin becomes present on the platelet surface. Once present, P-selectin is able to interact with sulfatides which triggers an internal phosphorylation pathway thought to be

mediated by p38 (Figure 3.1A) [24]. This cascade results in further degranulation and an increase in surface P-selectin [24]. To monitor the degranulation of platelets two activation markers were evaluated using fluorescent antibodies specific for P-selectin and the $\alpha_{IIb}\beta_3$ integrin. Marker surface expression was quantified using flow cytometry analysis, and measuring the median fluorescent signal of 10,000 platelets. The median signal was used as our reference to quantify the data set as median values are less affected by the wide range of platelet signals and is therefore more representative of the population. When platelets were stimulated with ADP (30 μM) they show increases in both surface P-selecting and $\alpha_{IIb}\beta_3$ integrin expression. In the presence of sulfatides (50μg / mL), in the form of sulfatide-enriched liposomes, the levels of P-selectin and $\alpha_{IIb}\beta_3$ integrin were further increased to about 20-25 fold their un-stimulated levels (Figure 3.1B). A stronger agonist TRAP (10 μM) however was able to induce complete degranulation, and the addition of sulfatides to TRAP stimulated platelets showed no affect on either P-selectin or $\alpha_{IIb}\beta_3$ integrin levels (Figure 3.1B). The levels of P-selectin and $\alpha_{IIb}\beta_3$ integrin were measured among several different individuals and the levels varied greatly between them, however consistently sulfatides induced a ~20 fold increase over un-stimulated platelets and a ~10 fold increase over ADP stimulated platelets.

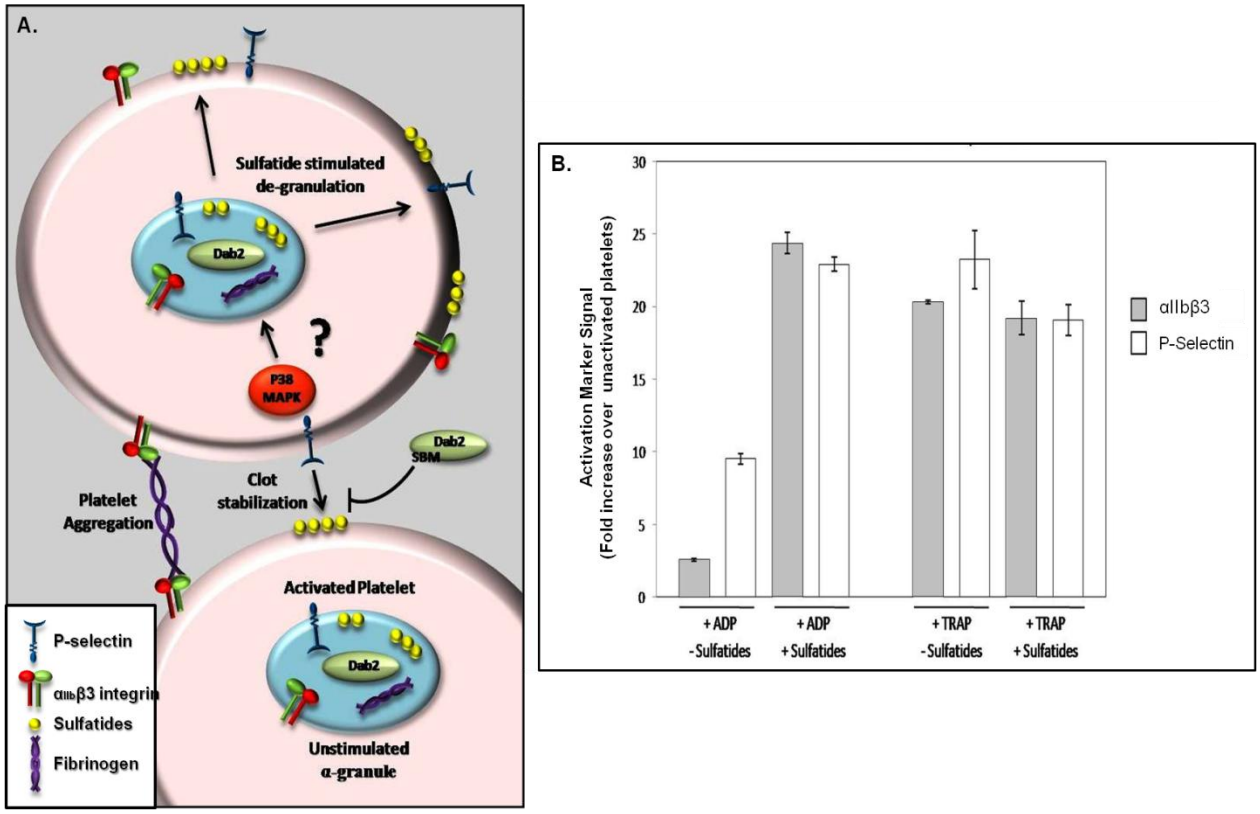


Figure 3.1 Sulfatide induced degranulation. **A**. P-selectin interaction with sulfatides on adjacent platelets stabilizes aggregates, as well as stimulating p38 mediated degranulation leading to increased surface P-selectin and $\alpha_{IIb}\beta_3$ integrin. **B.** The affect of sulfatides on surface P-selectin and $\alpha_{IIb}\beta_3$ integrin levels. Platelets treated with ADP (30μM) or TRAP (10 μM) were exposed to sulfatides (50 μg/mL) in the form of enriched liposomes, and changes in surface receptors were monitored using fluorescence signal detected by FLOW CYTOMETRY. P-selectin marker was PE-anti-human CD62P and the $\alpha_{IIb}\beta_3$ integrin marker was FITC-anti-human α_{IIb}. The median fluorescence was measured as a representative point of the population. Each bar in the bar graph represents the average of three separate reactions. The experiment was done multiple times and this is a representative

3.2 N-PTB sulfatide binding competes with P-selectin and inhibits surface expression of P-selectin and $\alpha_{IIb}\beta_3$ integrin.

During platelet activation Dab2 is released from α-granules and is able to bind to either the $\alpha_{IIb}\beta_3$ integrin or sulfatides [44, 18]. To define the role of Dab2 in the inhibition of sulfatide activation of platelets we monitored platelet surface P-

selectin and $\alpha_{IIb}\beta_3$ integrin in the presence of sulfatides and N-PTB wild type and mutants (Figure 3.2). Washed platelets were monitored for their levels of surface P-selectin using R-phycoerythrin (PE) labeled-anti-human P-selectin quantified using the median fluorescent signal detected by flow cytometry (Figure 3.2A). Un-treated washed platelets contain two populations, defined by the flow cytometry chromatogram, a peak at 10^1 PE signal defines low activation level while a second peak at 10^3 represents highly activated platelets (Figure 3.2B). The population of highly activated platelets in the untreated population most likely results from activation triggered by the washing process. ADP stimulation of the platelets results in a shift and broadening of the peak corresponding to low activated platelets, resulting in an increased median fluorescence (Figure 3.2A-B). The addition of control liposomes, liposomes that do not contain sulfatides, to ADP stimulated platelets did not trigger any additional shift in the peak and the median fluorescence remained consistent with ADP treatment (Figure 3.A-B).

Upon the addition of sulfatides to ADP stimulated platelets there was a complete shift of the low activation peak to the highly activated population (Figure 3.2C). This shift resulted in a roughly 12 fold increase in the median fluorescent signal compared to ADP treated platelets (Figure 3.2-1 A). The addition of N-PTB to the washed platelets was able to outcompete the P-selectin for sulfatide binding and therefore prevent the activation of the platelets. The low activation population

was unaffected in the presence of N-PTB and the median fluorescent signal remained at ADP stimulated levels (Figure 3.2-1 A-C).

To determine the role of $\alpha_{IIb}\beta_3$ integrin binding ability of N-PTB sulfatide inhibition was measured using mutant N-PTB. N-PTB^{4M} is unable to bind to sulfatides, but inhibits fibrinogen binding to the $\alpha_{IIb}\beta_3$ integrin [18]. However, N-PTB^{4M} showed no inhibition of sulfatide induced activation (Figure 3.2-1 A-C). N-PTB^{D66E} is unable to bind to the $\alpha_{IIb}\beta_3$ integrin but is still able to interact with sulfatides, and therefore showed complete inhibition of sulfatide induced activation (Figure 3.2-1 A-C). N-PTB^{5M}, N-PTB containing both the 4M and D66E mutations, cannot interact with either sulfatides or the $\alpha_{IIb}\beta_3$ integrin and shows no affect on sulfatide induced activation (Figure 3.2-1 A-C).

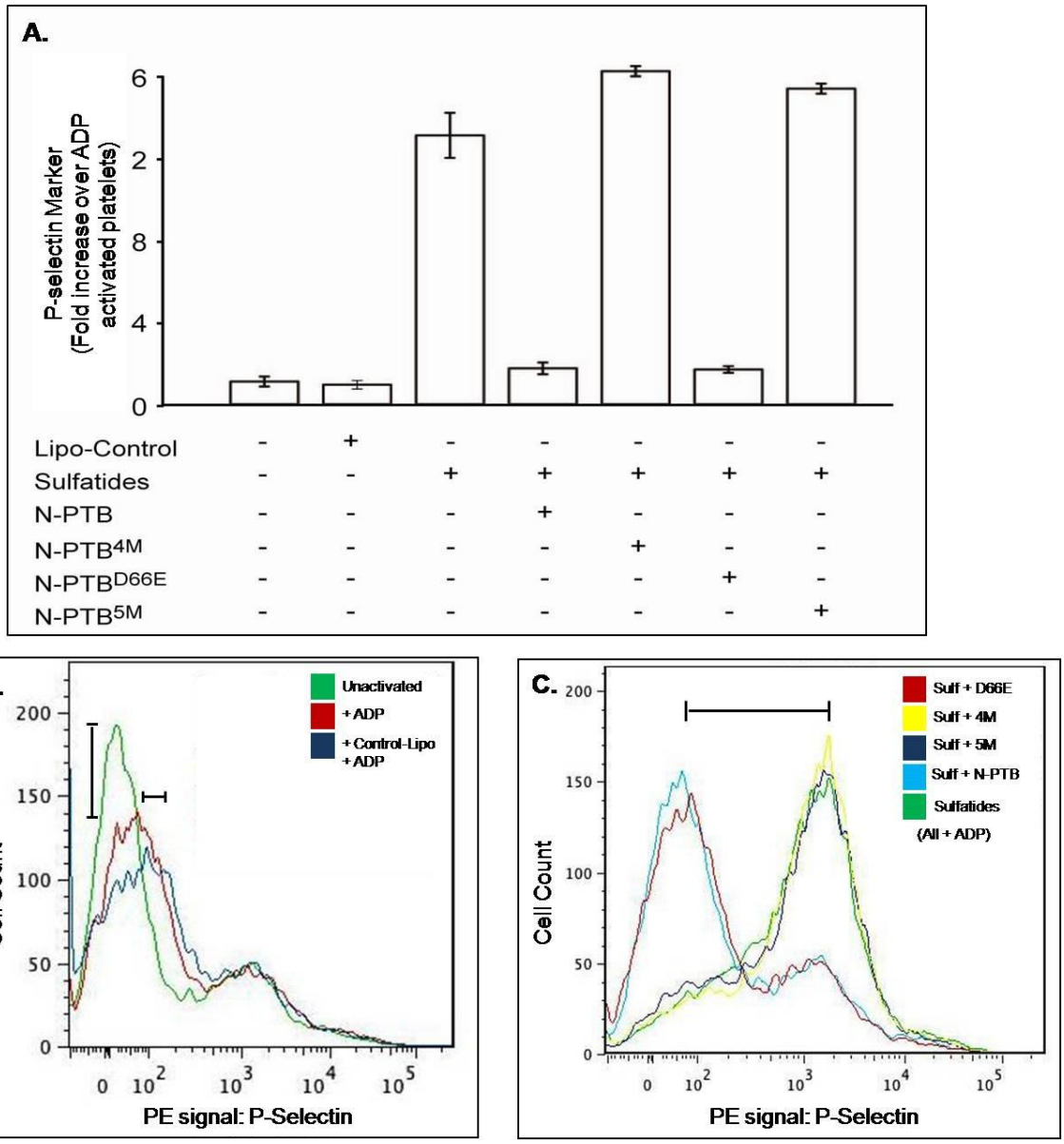


Figure 3.2-1 N-PTB inhibition of sulfatide induced degranulation. Platelets were either left unactivated or stimulated with ADP (30 μM). ADP stimulated platelets were also treated with either sulfatide (50 μg/mL) enriched liposomes (sulfatides) or un-enriched liposomes (Control-Lipo). Platelets were stimulated with sulfatides in the presence of N-PTB or N-PTB4M. After incubation for 6 minutes the reaction was fixed and P-selectin marker was added. **A.** Graph of the median fluorescence of P-selectin marker on the surface of platelets. Platelets are treated with ADP, either control or sulfatide liposomes, and different mutant constructs of N-PTB. **B and C.** Representative chromatograms of the fluorescent signal detected for each reaction. Chromatograms show the fluorescent signal for each platelet detected and shifts in the peaks represent changes in the marker presence. **B.** Black bars represent the shift of ADP stimulated platelets and control –lipo + ADP platelets. **C.** A black bar represents the shift of sulfatide stimulated platelets in the presence of different N-PTB constructs. Sulfatide stimulation results in a shift right, while inhibition prevents any shift.

Sulfatide induced activation also increases surface $\alpha_{IIb}\beta_3$ integrin through α-granule degranulation. Surface $\alpha_{IIb}\beta_3$ integrin was monitored using FITC-anti-human $\alpha_{IIb}\beta_3$ integrin, and quantified using median fluorescence signal detected by flow cytometry. Resting platelets show a peak of fluorescence below 10^1, the addition of ADP shifts the peak to above 10^2 (Figure 3.2-2 B). The addition of ADP stimulated not only the release of the $\alpha_{IIb}\beta_3$ integrin but also the activation of the $\alpha_{IIb}\beta_3$ integrin that is constitutively expressed on the surface, leading to a stronger response than the ADP affect on P-selectin. The addition of sulfatides further increases the fluorescence peak and causes a 2.5 fold increase in the median fluorescence (Figure 3.2-2 A-C). N-PTB inhibition of sulfatide induced expression of $\alpha_{IIb}\beta_3$ integrin is dependent upon sulfatide binding as N-PTB^{4M} showed no inhibition (Figure 3.2-2 A-C).

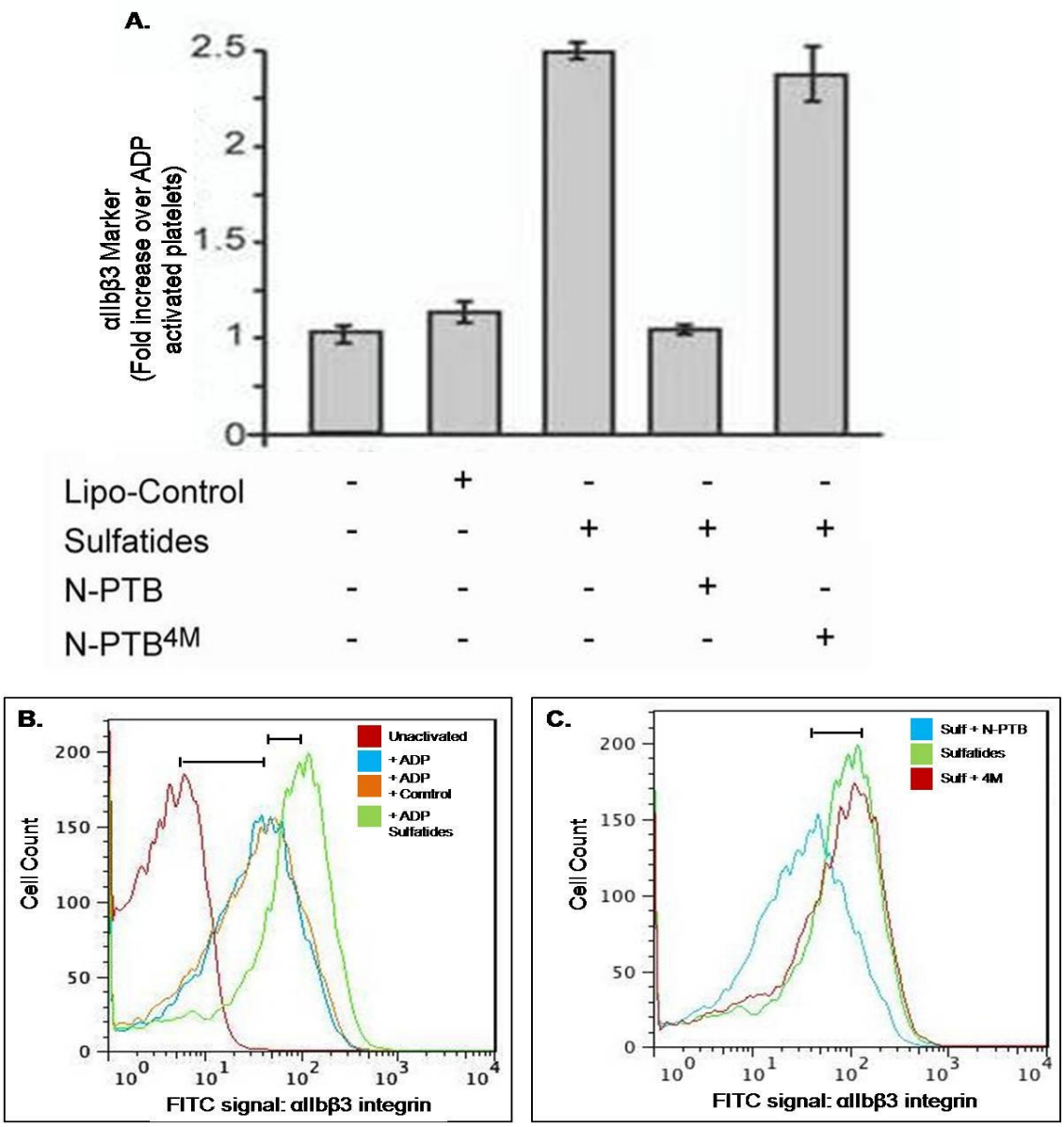


Figure 3.2-2 N-PTB inhibition of sulfatide induced degranulation. Platelets were either left unactivated or stimulated with ADP (30 μM). ADP stimulated platelets were also treated with either sulfatide (50 μg/mL) enriched liposomes (sulfatides) or un-enriched liposomes (Control-Lipo). Platelets were stimulated with sulfatides in the presence of N-PTB or N-PTB4M. After incubation for 6 minutes the reaction was fixed and $\alpha_{IIb}\beta_3$ integrin marker was added. **A.** Graph of the median fluorescence of P-selectin marker on the surface of platelets. Platelets are treated with ADP, either control or sulfatide liposomes, and different mutant constructs of N-PTB. **B and C.** Representative chromatograms of the fluorescent signal detected for each reaction. Chromatograms show the fluorescent signal for each platelet detected and shifts in the peaks represent changes in the marker presence. **B.** Black bars represent the shift of ADP stimulated platelets and control –lipo + ADP platelets. **C.** A black bar represents the shift of sulfatdie stimulated platelets in the presence of different N-PTB constructs. Sulfatide stimulation results in a shift right, while inhibition by N-PTB prevents any shift.

3.3 N-PTB binding of $\alpha_{IIb}\beta_3$ integrin does not influence sulfatide inhibition.

Dab2 splits into two pools during platelet aggregation, $\alpha_{IIb}\beta_3$ integrin bound and sulfatide-bound states. When sulfatide binding is abolished an increase in $\alpha_{IIb}\beta_3$ integrin binding is observed [18]. To determine whether N-PTB inhibition of sulfatide activation is increased with the abolition of $\alpha_{IIb}\beta_3$ integrin binding, titrations of both N-PTB and N-PTB^{D66E} and an IC_{50} value was calculated (Figure 3.3).

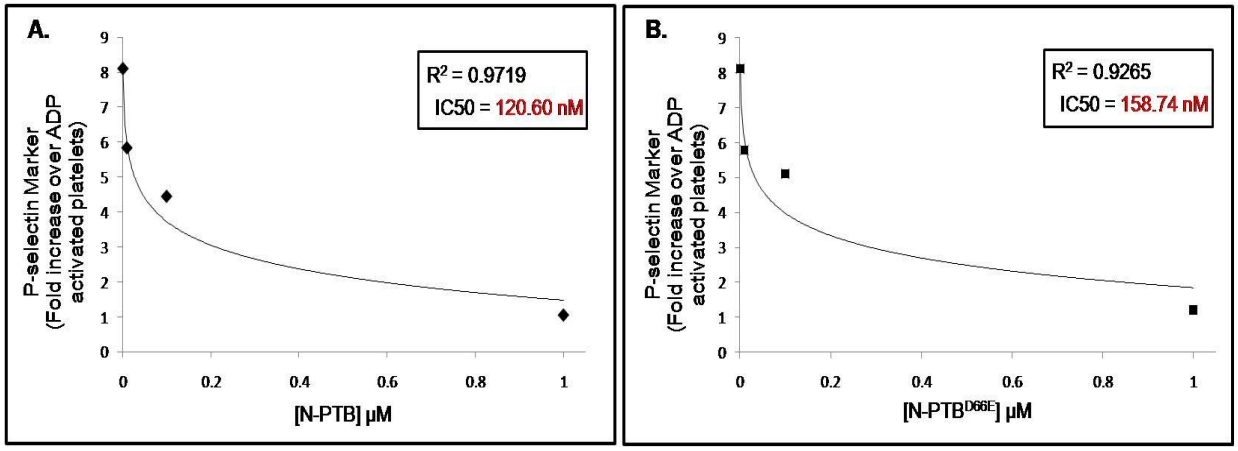


Figure 3.3 IC_{50} curves of N-PTB and N-PTB^{D66E} titrations. **A and B.** Titration curves of increasing concentrations of N-PTB and N-PTB^{D66E}. Increasing amounts of N-PTB and N-PTB D66E (1 nM - 1µM) were incubated with platelets before the addition of sulfatide enriched liposomes as previously described. The resulting P-selectin marker signals were detected by FLOW CYTOMETRY and plotted as a titration curve. Inhibition of sulfatides was calculated using sulfatide induced P-selectin marker expression, with complete inhibition being considered when P-selectin levels return to ADP stimulated platelets. Logarithmic curves were used to model the inhibition and IC_{50} values were calculated using the formulas produced by the curves.

The titration curves matched a logarithmic model with a correlation greater than 0.9 for both curves. The calculated IC_{50} values were 120.60 nM for N-PTB (Figure 3.3A), and 158.74 nM for N-PTB^{D66E}(Figure 3.3B). These values indicate that when the $\alpha_{IIb}\beta_3$ integrin bound pool of N-PTB shifts to become available for sulfatide binding the inhibitory affect of Dab2 is not changed. We hypothesize that this may be because the integrin bound pool is significantly smaller than the sulfatide pool, and thus the shift doesn't significantly affect sulfatide binding.

3.4 N-PTB is able to prevent sulfatide induced platelet aggregation under physiological flow conditions.

Platelet aggregation is mediated by $\alpha_{IIb}\beta_3$ integrin interactions with fibrinogen and fibrin linking platelets together [9, 10]. P-selectin links aggregated platelets together stabilizing clot formation [34]. Sulfatides stimulate increased levels of both $\alpha_{IIb}\beta_3$ integrin and P-selectin expression on platelets [24]. To observe platelet aggregation under flow conditions platelet's were flown through a microfluidics channel with a constant velocity and observed using light microscopy. The channel dimensions are 30 x 0.5 x 0.05 mm (length x width x height). Washed platelets were flown, at a shear rate of 70 s^{-1}, within the physiological range of capillary blood flow, through a channel coated with adhesive protein. The channel was coated by the incubation of human plasma, which contains soluble vWF, fibrinogen, and other adhesive proteins, in the channel for 2 hours allowing for the

protein to adhere to the glass slide. Platelets would become activated by interacting with the adhesive proteins, and platelet-platelet interactions facilitated aggregate growth. Platelets were not previously stimulated with ADP as we would allow for physiological activation of the platelets through interactions with the channel's adhesive proteins. Platelets, untreated or treated with sulfatide or control liposomes, were flown through the chamber for 10 minutes and pictures were taken at 0, 3, and 10 minutes, based on our pervious observations of Dab2 functioning in platelet aggregation [18]. Untreated and control liposome treated platelets showed activation and adhesion based on our observations of platelet monolayer formation, and low levels of aggregate formation (Figure 3.4). Quantitative analysis of the adhesion and rate of aggregation were done in collaboration with Dr, Pavlos Vlachos (Figure 6.2). Sulfatide liposomes stimulated much larger platelet aggregates than both untreated and control liposomes, observed qualitatively and quantified in collaboration (see Figure 6.2, 6.3) (Figure 3.4). N-PTB was added along with sulfatide liposomes in order to constrain sulfatide inducted aggregation, and with N-PTB (10 μM) the platelet aggregates returned to the size of untreated platelets (Figure 3.4). N-PTB^{4M} (10 μM) showed limited inhibition of platelet aggregate size but not adhesion (see Figure 6.2, 6.3), we hypothesize this is due to the increased binding to the $\alpha_{IIb}\beta_3$ integrin which limits platelet-platelet aggregation but not adhesion [18, 44].

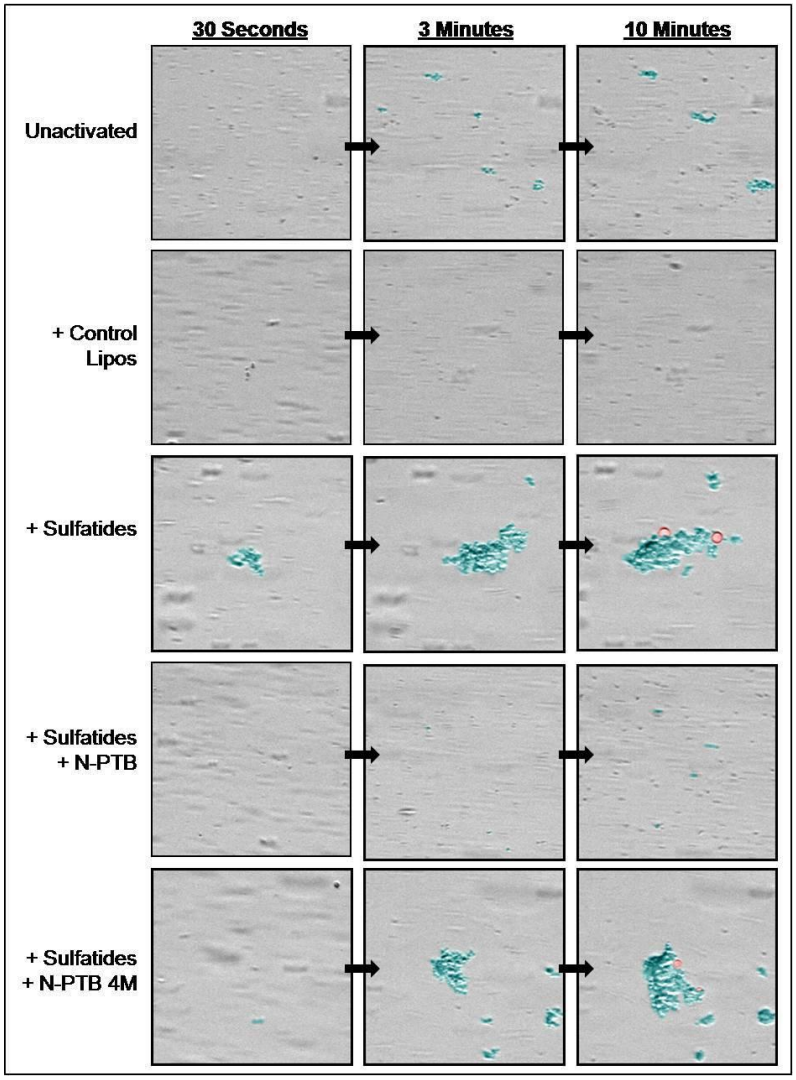


Figure 3.4 Platelet Aggregation under flow assay. Platelets were either left untreated or incubated with either N-PTB or N-PTB4M. The platelets were then mixed with either sulfatide enriched liposomes, or un-enriched liposomes and immediately pumped through the microfluidics channel at a shear rate of 70 s^{-1}. The channel was coated with soluble adhesive proteins from human plasma. Platelet adhesion and aggregation was monitored using bright field microscopy, and pictures of the channel were taken at 0, 3, and 10 minutes. Representative clots are shown.

3.5 N-PTB sulfatide binding limits platelet-leukocyte interactions.

Platelet P-selectin mediates platelet-leukocyte interactions through binding of leukocyte PSGL-1 [24]. Sulfatide stimulation of platelets results in increased levels of P-selectin expression, and N-PTB is able to inhibit this stimulation (Figure 3.5 A). Sulfatides are also able to activate leukocytes directly through binding to L-selectin and an L-selectin independent pathway (Figure 3.5A) [41].

To determine sulfatide and N-PTB's role in platelet-leukocyte interactions, platelet and leukocyte mixtures (10^8 platelets/mL, 10^7 leukocytes/mL) were activated with ADP (30 μM) and incubated with control or sulfatide liposomes (50 μg/mL). Platelet binding of leukocytes was quantified through the fluorescence of allophycocyanin (APC) labeled anti-human CD42b (platelet marker) detected on leukocytes. The fluorescence was detected using flow cytometry analysis of platelet-leukocyte mixtures.

Platelet stimulation with ADP led to a very slight increase in platelet-leukocyte interactions corresponding to a slight increase in P-selectin (Figure 3.5C). Addition of sulfatides resulted in a 3-fold increase in platelet-leukocyte interactions (Figure 3.5B), the flow cytometry chromatogram shows a positive shift in the fluorescence (Figure 3.5C). The addition of N-PTB decreased platelet binding to leukocytes, seen by a decrease in median fluorescence signal as well as a negative shift of the fluorescence peak (Figure 3.5A-D). N-PTB^{4M} showed no inhibition of platelet-leukocyte binding.

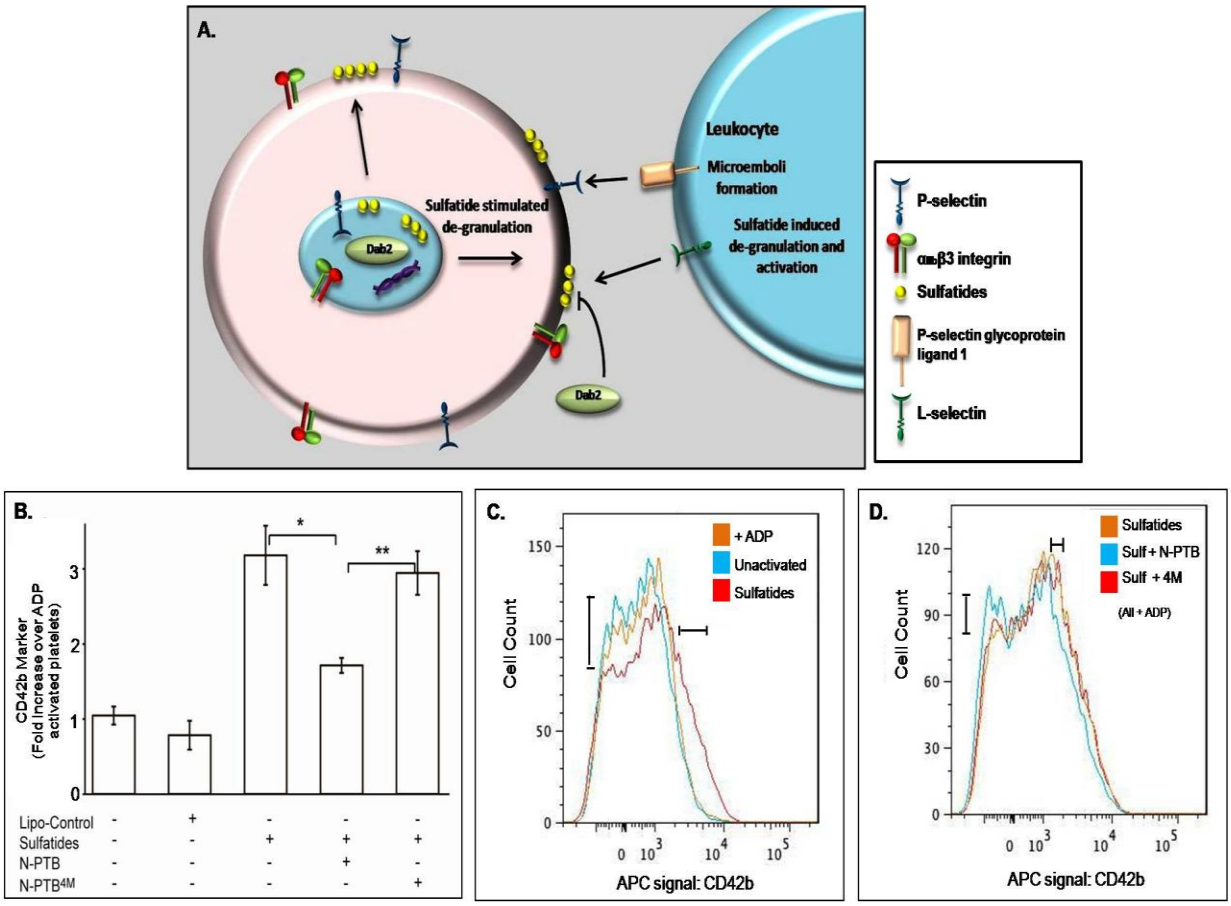


Figure 3.5 Platelet-leukocyte binding is mediated by sulfatides. **A.** Sulfatides stimulate platelet P-selectin as well as stimulating leukocytes directly, Dab2 is able to inhibit platelet-leukocyte aggregation through sulfatide binding. **B.** Platelet and leukocyte mixtures (10^8 platelets/ mL and 10^7 leukocytes/mL) were incubated with either N-PTB or N-PTB4M. Liposomes, either sulfatide enriched or not, were added to the mixtures stimulated with ADP (30 μM). Reactions incubate for 6 minutes and are fixed and APC-anit-human CD42b is added. The Cd42b fluorescence is quantified using FLOW CYTOMETRY analysis. Leukocytes are identified based on their forward and side scatter plots as distinctive from platlets. Graph of the median fluorescence signal of platelet marker CD42b detected in the leukocyte population represents platelet-leukocyte interactions. **C and D.** Flow cytometry chromatograms showing the fluorescence of CD42b within the leukocytes for each treatment, black bars represent the resulting shifts in platelet signal.

3.6 N-PTB ablates platelet-leukocyte binding under flow conditions.

To quantify the ability of N-PTB to block platelet-leukocyte binding under physiological flow conditions, platelet and leukocyte mixtures were flown through a microfluidics device, as described above, and aggregation was measured using light microscopy. Untreated cell suspensions (10^8 platelets/mL, 10^7 leukocytes/mL) were flown for 10 minutes and the channel was scanned for leukocyte adhesion and inclusion into platelet aggregates and representative pictures were taken (Figure 3.6A-B). The addition of sulfatides showed a marked increase in adhesive leukocytes and platelet-leukocyte clot size over untreated and control liposome treated samples (Figure 3.6A).

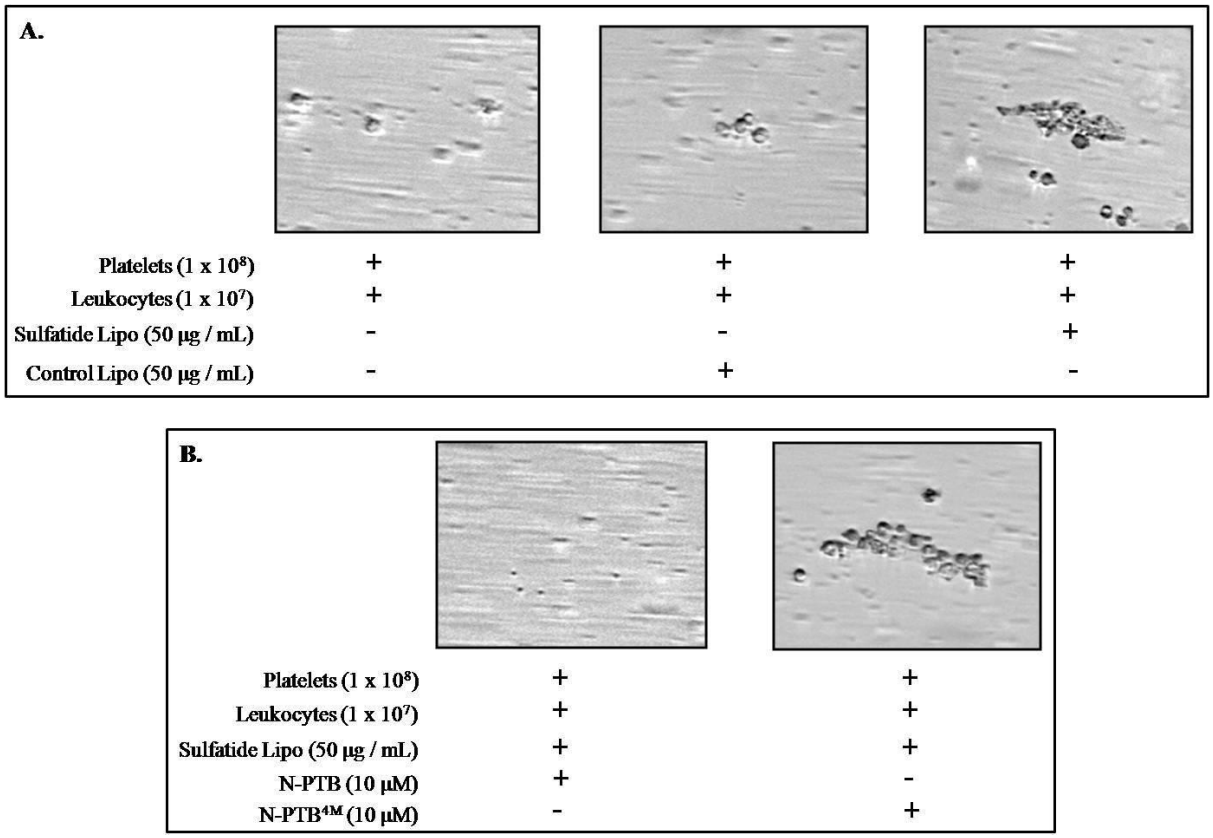


Figure 3.6 Platelet-leukocyte aggregation under flow. Platelet-leukocyte mixtures (10^8 platelets/mL and 10^7 leukocytes/mL) were flown through a microfluidics channel. The channel was coated with adhesive proteins and the flow produced a shear rate of 70 s^{-1}. Platelet-leukocyte aggregates were monitored using bright field microscopy throughout the channel after 10 minutes of stead flow. **A.** Untreated, as well as control and sulfatide liposome treated cell suspensions were flown through the channel for 10 minutes, and representative aggregate pictures are shown. **B.** Platelet-leukocyte mixtures containing sulfatide liposomes and either N-PTB or N-PTB^{4M} (10 µM) were flown for 10 minutes, and representative aggregate pictures are shown.

When N-PTB (10 µM) was added there was a great reduction in observed leukocyte adhesion as well as platelet-leukocyte aggregates (Figure 3.6B). N-PTB^{4M} was unable to affect the adhesion and aggregation of leukocytes.

Chapter 4: Discussion and Conclusions

Platelet aggregation serves as a response to vascular injury by, stopping bleeding, mediating the inflammatory response, and to aid in wound healing. Sulfatides present in growing clots increase aggregation by inducing degranulation. This serves to enhance platelet aggregation through increasing surface $\alpha_{IIb}\beta_3$ integrin which mediates platelet-platelet interactions, and P-selectin which stabilizes platelet aggregates and facilitates platelet-leukocyte interactions. Upon degranulation Dab2 is released and inhibits platelet aggregation through $\alpha_{IIb}\beta_3$ integrin and sulfatide binding [44, 18]. Dab2 is capable of competing directly with fibrinogen for $\alpha_{IIb}\beta_3$ integrin, however binding to sulfatides prevents integrin binding. In order to determine the role of Dab2-sulfatide binding in platelet aggregation we monitored the presence of degranulation markers on platelets in the presence of sulfatide enriched liposomes. The addition of N-PTB to platelets stimulated with ADP and exposed to sulfatide-enriched liposomes was able to completely eliminate secondary degranulation (Figure 3.2-1 and 3.2-2). Platelet $\alpha_{IIb}\beta_3$ integrin and P-selectin remained at ADP stimulated levels in the presence of both N-PTB, and N-PTB^{D66E}, both of which maintain their ability to bind to sulfatides. N-PTB^{4M} and N-PTB^{5M}, both contain mutations to the sulfatide binding residues and therefore show no inhibition of secondary degranulation. This shows that the integrin binding ability of Dab2 plays no role in inhibiting platelet

secondary activation through sulfatide binding. Upon the release of Dab2 from α-granules the literature estimated concentration was approximately 1-2 μM. The calculated IC50 of N-PTB on sulfatide stimulated degranulation is ~120 nM well within the estimated physiological levels.

To observe the affect of platelets under flow conditions a microfluidics aggregation assay was established. A glass cover slip coated with soluble proteins from human plasma is placed over a channel. Un-treated platelets and platelets treated with control liposomes showed adhesion and slight secondary aggregation, quantitative analysis showed that these treatments resulted in many adhesive platelets and small cluster sizes (Figure 3.4). When sulfatide enriched liposomes were introduced secondary aggregation resulted in much larger aggregates. The addition of N-PTB^{4M} was unable to prevent secondary aggregation, but analysis of the size of the largest cluster, mean cluster size, and total cluster coverage showed that N-PTB^{4M} did reduce the size of the aggregates. Interestingly N-PTB^{4M} treated platelets showed the same cluster number as platelets alone when exposed to sulfatides. This supports the purposed model that $\alpha_{IIb}\beta_3$ integrin binding inhibits platelet-platelet aggregation reducing cluster size, but is unable to affect adhesion mediated through other surface proteins so cluster number is the same. Addition of N-PTB to sulfatide treated platelets lead to inhibited platelet adhesion as well as aggregation. Both number of clusters and cluster size were reduced below other

sulfatide treated reactions. This again supports the model of Dab2 as a dual inhibitor of aggregation though both $\alpha_{IIb}\beta_3$ integrin binding as well as sulfatide binding.

Platelet P-selectin stabilizes clot formation but it also serves as the main receptor responsible for platelet-leukocyte binding. P-selectin binds to PSGL-1 present on the leukocyte cell surface. Sulfatide induced degranulation greatly increases surface P-selectin expression therefore increasing platelet-leukocyte binding. Dab2 inhibition of sulfatide binding prevents secondary degranulation of P-selectin and therefore platelet-leukocyte binding (Figure 3.5 and 3.6). ADP stimulation of circulating platelets causes the activation of constitutively expressed $\alpha_{IIb}\beta_3$ integrin as well as low levels of degranulation. The surface of the platelet has a higher ratio of $\alpha_{IIb}\beta_3$ integrin to P-selectin than a completely de-granulated platelet which promotes platelet-platelet interactions over platelet-leukocyte interactions. The addition of sulfatides to platelet-leukocyte mixtures resulted in a three fold increase in binding. Sulfatide degranulation therefore serves as mechanism to ensure that clot incorporated platelets express high levels of P-selectin allowing for the recruitment of leukocytes. N-PTB limited the aggregation through sulfatide binding, while N-PTB^{4M} showed no inhibition of binding. Therefore Dab2 when released from α-granules binds to sulfatides and blocks the expression of P-selectin inhibiting the recruitment of leukocytes to the platelet

aggregate. The release of Dab2 extracellularly inhibits platelet homotypic and heterotypic aggregation through a dual mechanism, one pool binds to surface $\alpha_{IIb}\beta_3$ integrin limited platelet-platelet interactions, and another pool which binds to sulfatides inhibiting secondary activation leading to clot stability and leukocyte recruitment (Figure 4.1).

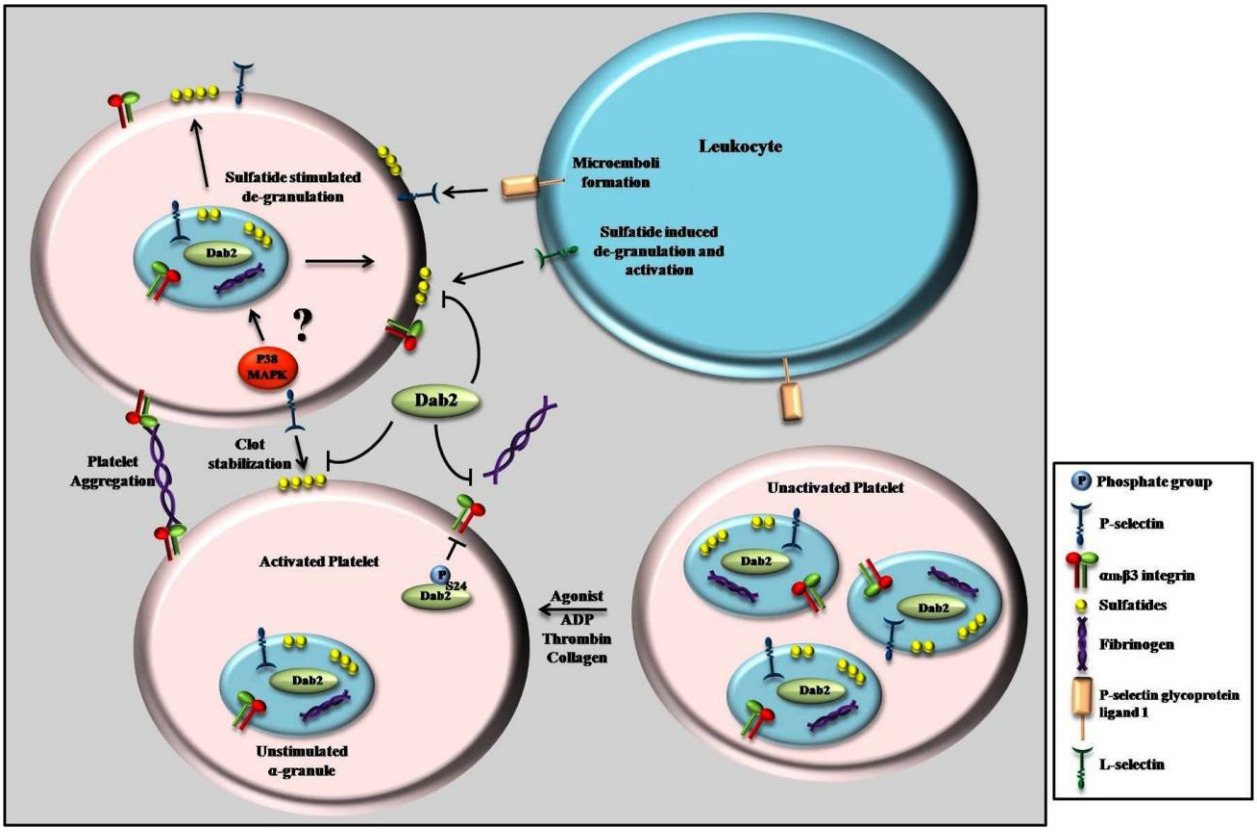


Figure 4.1 The role of Dab2 in platelet aggregation. Dab2 inhibits the $\alpha_{IIb}\beta_3$ integrin both intracellularly and extracellularly. Dab2 binding of sulfatides inhibits platelet-sulfatide interactions which decreases clot stability and blocks degranulation. Dab2 inhibition of degranulation results in decreased P-selectin expression. Decreased P-selectin and blocking sulfatides from stimulating leukocytes results in decreased platelet-leukocyte interactions.

Chapter 5 Materials and Methods

Liposome preparation

Stocks of brain sulfatides, phosphatidylcholine (PC), phosphatidylethanolamine (PE) (Avanti Polar Lipids) and cholesterol (Sigma) were prepared in organic solvents per manufacturer instructions. Liposomes were prepared in the absence and presence of sulfatides as described (5).

Blood collection and platelet purification

Whole blood was collected from healthy volunteers by venipuncture into 10% acid citrate dextrose blood collection tubes. Whole blood was then centrifuged at 200xg for 15 min to separate platelet rich plasma (PRP) from contaminating erythrocytes. PRP was removed and centrifuged at 2,200xg for 10 min to remove platelets from the plasma. Platelet poor plasma was

removed and platelets suspended in Tyrode's albumin buffer (10 mM HEPES (pH 7.4), 134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.34 mM Na2HPO4 and 1 mM MgCl2,) containing 10 U/mL heparin and 0.5 μM prostaglandin (PGI2). Platelets were washed again in Tyrode's albumin buffer containing PGI2 and counted.

Device design

The microfluidic device consisted in a simple straight channel 500 μm wide, 50 μm deep and 3.62 cm long. A silicon master stamp was fabricated on a <100> silicon substrate following our previously described process (8). Flow was driven using a micro-syringe pump (Cole-Parmer) with a 1 mL syringe connected to the channel inlet by 30 cm of Cole-Parmer gauge 20 Teflon
tubing. After priming the system with the sample, the pump was set to 0.05 mL/h (equivalent to an average velocity of 0.55mm/sec in the channel), which is in the range of in vivo blood velocity (0.1-1.5 mm/sec (9)) and causes a shear rate of 70 sec-1. This flow rate was maintained for 1 min prior to the experiments. Platelets flowing through the channel were monitored using an inverted light microscope (DMI 6000B, Leica Microsystems) equipped with a digital camera (DFC420, Leica Microsystems).

Flow cytometry

Washed platelets (2.5 x 105 platelets/μL) were kept unactivated or either activated with ADP (30 μM) or TRAP (10 μM). Both unactivated and activated platelets were incubated for 10 min at 23°C with liposomes (50 μg/mL) either without (liposomes control) or with sulfatides, and N-PTB constructs (1 μM). Reactions were fixed with 1% formalin and incubated with PE- labeled CD62p anti-P-

selectin (BioLegend) or FITC-labeled PAC-1 anti-integrin receptor (BD Transduction) antibodies. Bound antibodies were quantified using a Flow cytometryAria flow cytometer.

Leukocyte purification and platelet-leukocyte aggregation assay

Whole blood was collected as indicated above and centrifuged at 200xg to separate the blood from plasma and red blood cell fractions. Plasma and buffy coat layer were carefully removed and diluted 1:1 with PBS. The dilution was then layered onto a Ficoll Plus gradient and spun at 200xg for 20 min. The enriched platelet and leukocyte layer was removed, diluted with PBS, centrifuged and the pellet resuspended in Tyrode's Albumin buffer to a concentration of ~3 x 105 platelets/μL and 3 x 103 leukocytes/μL. Isolated platelet-leukocyte mixtures were remained unactivated or activated with ADP (30 μM). Both unactivated and activated platelets were incubated for 10 min at 23°C with liposomes (50 μg/mL) either without (liposomes control) or with sulfatides, and N-PTB constructs (1 μM). Reactions were fixed with 1% formalin and incubated with APC-labeled CD42b (Biolegend) and FITC-labeled CD45 (Biolegend) antibodies. Bound antibodies were quantified using a Flow cytometryAria flow cytometer.

Chapter 6: Collaborations

A. Sulfatides Partition Disabled-2 in Response to Platelet Activation. Karen E. Drahos, John D. Welsh, Carla V. Finkielstein,and Daniel GS. Capelluto. November 2009, *PLoS ONE*. 4(11).

Contributions I have made to this project are stated in the Acknowledgements section of
the publication (see attached):

"Conceived and designed the experiments: KED JDW cf DGC. Performed the experiments: KED JDW. Analyzed the data: KED JDW cf DGC. Contributed reagents/materials/analysis tools: cf DGC. Wrote the paper: cf DGC. Performed surface plasmon resonance, lipid-protein overlay assay, circular dichroism, immunofluorescence analysis, and pull-down experiments: KED. Designed and performed the liposome binding and flow cytometry assays: KED JDW. Performed thrombin digestion and cell adhesion assays: JDW."

B. Quantitative analysis of Disabled-2 inhibition of platelet aggregation. John Charonko, John D. Welsh, Alireza Salmanzadeh-Dozdabi, Carla V. Finkielstein, and Pavlos P. Vlachos. *In preparation.*

Disabled-2 (Dab2) is released upon platelet activation and inhibits platelet aggregation through binding to the α_{IIb} subunit of the $\alpha_{IIb}\beta_3$ integrin as well as sulfatides. Using platelet aggregation assays, and mutant constructs of the N-PTB region of Dab2 we are able to demonstrate the physiological effects of $\alpha_{IIb}\beta_3$ integrin binding and sulfatide binding in platelet aggregation. We flowed platelets through a microfluidics channel coated with adhesive proteins, and clot formation was monitored using light microscopy. Clot formation was monitored with 10 minutes of video recording (12 frames / second). By quantitative analysis of the size, rate of formation, and adhesion of platelets and platelet aggregates we can analyze the affect of different Dab2 binding partners on platelet physiology.

J. C. performed the quantitative analysis, A. S-D and J.W. performed the aggregation assays. C.V.F. and P.V. directed the project. All of the authors contributed to the intellectual development of the project.

Case name:	"no treatment"	"+ control"	"+ sulfatides"	"4M alone"	"PTB alone"	"sulf + 4M"	"sulf+PTB"
Platelets	+	+	+	+	+	+	+
Liposomes		+	+			+	+
Sulfatides			+			+	+
4M				+		+	
N-PTB					+		+
Expected response:	monolayer	monolayer	clot	Inhibited adhesion	Inhibited adhesion	clot	No clot
Apparent response:	scattered adhesion	scattered adhesion	large aggregates	scattered adhesion	Minimal adhesion	large aggregates	minimal adhesion

Figure 6.1 Qualitative analysis of platelet aggregation. Platelets (3×10^8 were treated with sulfatide or control liposomes, N-PTB wild type and 4M, or untreated. Platelet aggregation was monitored under flow conditions using light microscopy for 10 minutes. Qualitative observations of clot formation are given above for each of the platelet treatments.

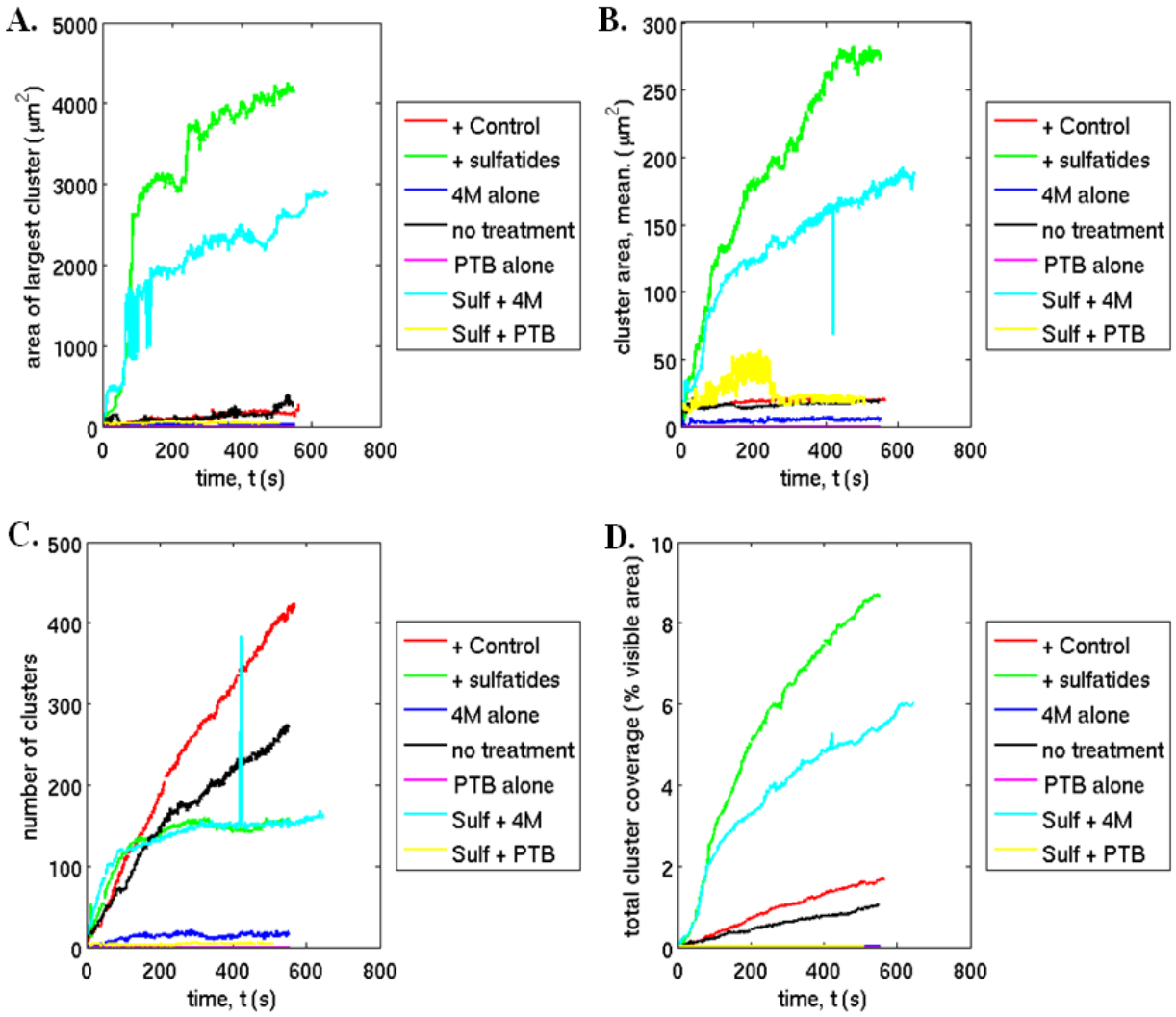


Figure 6.2 Quantitative analysis of platelet aggregation over time. Quantification of the area of the largest cluster, average cluster area, total number of clusters, and total cluster coverage were measured over time. After subtracting the background from the initial frame all platelets were identified as either moving or stationary. Stationary platelet clusters are identified and their growth monitored over time for all clusters. The size is determined based on pixilation of the cluster, and the largest cluster after 10 minutes is identified and its growth rate plotted over time. All the clusters are quantified and the average size is calculated and plotted over time. The total number of clusters and the visible area were quantified and plotted against time.

Appendix:

IRB Approval CF-08-196-IR

-April 1st 2008-2009

-April 1st 2009-2010

VirginiaTech

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www.irb.vt.edu
FWA00000572(expires 1/20/2010)
IRB # is IRB00000667

DATE: April 2, 2008

MEMORANDUM

TO: Carla Finkielstein
Karen Drahos
John Welsh

FROM: David M. Moore

Approval date: 4/2/2008
Continuing Review Due Date:3/18/2009
Expiration Date: 4/1/2009

SUBJECT: **IRB Expedited Approval:** "A Novel Ligand Drives Lipid Competition for the Disabled-2 PID Domain" , IRB # 08-196

This memo is regarding the above-mentioned protocol. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. As Chair of the Virginia Tech Institutional Review Board, I have granted approval to the study for a period of 12 months, effective April 2, 2008.

As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
3. Report promptly to the IRB of the study's closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher's responsibility to obtain re-approval from the IRB before the study's expiration date.
4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

Important:
If you are conducting **federally funded non-exempt research**, please send the applicable OSP/grant proposal to the IRB office, once available. OSP funds may not be released until the IRB has compared and found consistent the proposal and related IRB applicaton.

cc: File

VirginiaTech

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FWA00000572(expires 1/20/2010)
IRB # is IRB00000667

DATE: March 11, 2009

MEMORANDUM

TO: Carla Finkielstein
Karen Drahos
John Welsh

FROM: David M. Moore

Approval date: 4/2/2009
Continuing Review Due Date:3/18/2010
Expiration Date: 4/1/2010

SUBJECT: **IRB Expedited Continuation 1:** "A Novel Ligand Drives Lipid Competition for the Disabled-2 PID Domain" , IRB # 08-196

This memo is regarding the above referenced protocol which was previously granted expedited approval by the IRB. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. Pursuant to your request, as Chair of the Virginia Tech Institutional Review Board, I have granted approval for extension of the study for a period of 12 months, effective as of April 2, 2009.

Approval of your research by the IRB provides the appropriate review as required by federal and state laws regarding human subject research. As an investigator of human subjects, your responsibilities include the following:

1. Report promptly proposed changes in previously approved human subject research activities to the IRB, including changes to your study forms, procedures and investigators, regardless of how minor. The proposed changes must not be initiated without IRB review and approval, except where necessary to eliminate apparent immediate hazards to the subjects.
2. Report promptly to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.
3. Report promptly to the IRB of the study's closing (i.e., data collecting and data analysis complete at Virginia Tech). If the study is to continue past the expiration date (listed above), investigators must submit a request for continuing review prior to the continuing review due date (listed above). It is the researcher's responsibility to obtain re-approval from the IRB before the study's expiration date.
4. If re-approval is not obtained (unless the study has been reported to the IRB as closed) prior to the expiration date, all activities involving human subjects and data analysis must cease immediately, except where necessary to eliminate apparent immediate hazards to the subjects.

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46. Cerebroside Sulfotransferase Forms Homodimers in Living Cells† Afshin Yaghootfam,, Thomas Sorkalla,, Hanns Häberlein,, Volkmar Gieselmann,, Joachim Kappler, and, Matthias Eckhardt
Biochemistry 2007 *46* (32), 9260-9269