Platelets Possess and Require an Active Protein Palmitoylation Pathway for Agonist-Mediated Activation and In Vivo Thrombus Formation

Derek S. Sim, James R. Dilks, Robert Flaumenhaft

Objective—Several platelet proteins are palmitoylated, but whether protein palmitoylation functions in platelet activation is unknown. We sought to determine the role of platelet protein palmitoylation in platelet activation and thrombus formation.

Methods and Results—Platelet proteins were depalmitoylated by infusing acyl-protein thioesterase 1 into permeabilized platelets. In intact platelets, platelet protein palmitoylation was blocked using the protein palmitoylation inhibitor cerulein. The effects of inhibiting platelet protein palmitoylation on platelet function and on thrombus formation in vivo were evaluated. When infused into permeabilized platelets, acyl-protein thioesterase 1 reduced total platelet protein palmitoylation and inhibited protease-activated receptor-1–mediated α-granule secretion with an IC50 of 175 nmol/L and maximal inhibition of ≥90%. Gqα and SNAP-23, membrane-associated proteins that are constitutively palmitoylated, translocated to the cytosol when permeabilized platelets were exposed to recombinant acyl-protein thioesterase 1. The protein palmitoylation inhibitor cerulein also inhibited platelet granule secretion and aggregation. Studies using intravital microscopy showed that incubation with cerulein decreased the rate of platelet accumulation into thrombi formed after laser-induced injury of mouse arterioles and inhibited maximal platelet accumulation by >60%.

Conclusion—These studies show that platelets possess a protein palmitoylation machinery that is required for both platelet activation and platelet accumulation into thrombi. These studies show that inhibition of platelet protein palmitoylation blocks platelet aggregation and granule secretion. In a murine model of thrombus formation, inhibition of protein palmitoylation markedly inhibits platelet accumulation into thrombi at sites of vascular injury. (Arterioscler Thromb Vasc Biol. 2007;27:000–000-.)

Key Words: granule secretion ■ platelet ■ protein palmitoylation ■ signal transduction ■ thrombus formation

Protein palmitoylation involves the covalent linkage of a 16-carbon saturated fatty acid to a protein. Linkage of the 16-carbon fatty acid can occur via a thioester bond to a cysteine residue or through an amide linkage to a glycine or cysteine residue.1 Palmitoylation via a thioester bond, the more common form of palmitoylation, is reversible and regulated.2 Palmitoylation of proteins influences their association with membranes, enhances their incorporation into specific lipid domains such as rafts, and affects protein–protein interactions. Functional studies using either site-directed mutagenesis of relevant cysteine residues3,4 or palmitate analogues that inhibit protein palmitoylation5,6 demonstrate that protein palmitoylation can influence ligand-induced cell activation.

The observation that protein palmitoylation participates in activation-induced signal transduction implies a machinery capable of palmitoylating and depalmitoylating proteins. Genetic studies in yeast have identified palmitoyl transfer proteins that contain conserved DHHC–cysteine-rich domains sequences.6,7 Two human proteins, Golgi-specific DHHC zinc finger protein (GODZ) and Huntingtin-interacting protein (HIP) 14, containing DHHC–cysteine-rich domains sequences were subsequently identified and found to possess palmitoyl transfer activity.8,9,10 Palmitoylthioesterases are enzymes that remove palmitoyl moieties from proteins. Both palmitoyl protein thioesterases, which are localized primarily to lysosomes, and acyl-protein thioesterase 1 (APT1), which is localized primarily to cytosol, have been characterized.11,12 There is evidence that APT1 can function in signal transduction.13 The identification of both palmitoyl transfer proteins and palmitoylthioesterases demonstrates a means by which reversible posttranslational changes in protein palmitoylation may occur.

Many platelet proteins undergo palmitoylation. Platelet proteins that are palmitoylated include Gqα subunits,14 SNAP-23,15 glycoprotein (platelet glycoprotein) Ib,16 adenylyl cy-
Materials and Methods

Materials

All buffer constituents, streptolysoin-O (SL-O), cerulenin, protein A-Sepharose, and GTPγS, ADP, α-thrombin, and epinephrine were purchased from Sigma (St. Louis, Mo). 3H-palmitate was obtained from PerkinElmer (Boston, Mass). Collagen-related peptide was a generous gift from Dr Jonathan Gibbins. Collagen was obtained from Chrono-Log Inc. Calcein and calcein red-orange were purchased from Invitrogen (Carlsbad, Calif). Transfected Escherichia coli expressing His-tagged recombinant APT1 was kindly provided by Dr Thomas Michel, Brigham & Women’s Hospital (Boston, Mass).

Antibodies

Anti-APT1 antibodies were generated by immunizing rabbits with full-length recombinant APT1. Anti-SNAP-23 antibodies were generated by immunizing rabbits with a peptide corresponding to the C-terminus of SNAP-23 (DRIDIANARAKKLIDS). Antibodies were purified by affinity chromatography. Antibody specificity was confirmed by analysis of platelet lysates by SDS-PAGE. Anti-Gαi monoclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). Anti-GODZ antiserum was a generous gift from Dr Bernhard Luscher. Anti-HIP14 polyclonal antibody was a generous gift from Dr Michael Hayden.

Platelet Preparation

Platelets were purified from the blood of healthy donors using centrifugation and gel filtration as previously described. Platelets were purified into PIPES/NaCl buffer for experiments using intact platelets or in PIPES/EGTA/KCl buffer for experiments using permeabilized platelets.

Labeling Platelet Proteins With [3H]palmitate

Washed platelets (3×10^8/mL) were radiolabeled with 100 μCi/mL [3H]palmitate for 1 hour, unless otherwise indicated, in PIPES/NaCl buffer with 3.6 mg/mL bovine serum albumin in the presence or absence of the indicated reagents. Platelets were lysed in nonreducing sample buffer. [3H]palmitate-labeled platelet proteins were separated by SDS-PAGE, transferred onto a polyvinylidene fluoride membrane, exposed to a tritium detection screen, and analyzed using an Amersham Typhoon 9400 molecular imager.

Analysis of Platelet Cavitates

Washed platelets were incubated with the indicated concentrations of cerulenin at 37°C for 2 hours and tested for aggregation as previously described. Aggregation was initiated by SFFLRN, α-thrombin, ADP, epinephrine, collagen-related peptide, or collagen, and measured using a Chrono-Log 680 Aggregation System (Havertown, Pa).

Analysis of [3H]palmitoylated Gαi and SNAP-23

Washed platelets (2.5×10^8/mL) were radiolabeled with [3H]palmitate for 1 hour. Platelets were permeabilized by SL-O in the presence or absence of APT1 for 30 minutes and then lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate, pH 7.2, 1% Trasylol). After centrifugation of lysates, immunoprecipitation using 5 μg/mL anti-Gα or anti-SNAP-23 antibodies was performed with protein A-Sepharose beads by standard protocol. Immunoprecipitated proteins were separated by SDS-PAGE, transferred onto a polyvinylidene fluoride membrane, exposed to a tritium detection screen, and analyzed using a Typhoon 9400 molecular imager.

Preparation of Mice for Intravitral Microscopy

Six- to 8-week old C57BL/6 mice were anesthetized with an intraperitoneal injection of ketamine (125 mg/kg), xylazine (12.5 mg/kg), and atropine sulfate (0.25 mg/kg) and surgically prepared as previously described. The cremaster muscle was exposed and affixed over a glass slide to allow observation of the microcirculation in the muscle under an Olympus AX70 fluorescent microscope (Melville, NY). The cremaster microvasculature was viewed using a 40× water immersion lens. All procedures were approved by the Animal Care and Use Committee of the Beth Israel Deaconess Medical Center.

Analysis of Platelet Accrual at Sites of Vascular Injury Using Videomicroscopy

Washed mouse platelets were incubated with 500 μmol/L cerulenin or vehicle (0.2% DMSO) for 2 hours at 37°C. Cerulenin-treated platelets and control platelets were then washed and labeled for 30 minutes with calcine and calcine red-orange, respectively. Fluorescently labeled mouse platelets were washed and transfused through a cannulated jugular vein of mice so >0.3% of the circulating platelets were fluorescently labeled. Thrombi were induced by applying a pulsed nitrogen dye laser at 440 nm through the microscope objective using the Micropoint laser system (Photonics Instruments, St. Charles, Ill). The accumulation of cerulenin-treated platelets and vehicle-treated platelets into thrombi following laser ablation was recorded continuously for 5 minutes using digital videomicroscopy. Platelet accumulation into the thrombus over time was captured and analyzed using Slidebook software (Intelligent Imaging Innovations, Denver, Colo). After the experiment, a blood sample was obtained and the number of cerulenin-treated (calcine-labeled) and vehicle-treated (calcine red-orange–labeled) platelets counted by fluorescence microscopy to ensure that an equal amount of platelets were infused. For analysis, the number of platelets adhering to the thrombus was quantified by counting the number of cerulenin- and vehicle-treated platelets arrested at the site of vascular injury in 10-second intervals. Thirty-two thrombi generated in 3 mice were evaluated. Maximal platelet accumulation of cerulenin- and vehicle-treated platelets were compared for statistical analysis using the Wilcoxon rank sum test.

Evaluation of P-selectin Expression Using Flow Cytometry

Platelet permeabilization with SL-O has previously been described and characterized by our laboratory. Briefly, washed platelets were incubated in the presence or absence of APT1 and permeabilized with 500 U/mL SL-O at room temperature for 5 minutes. Permeabilized and intact platelets exposed to recombinant APT1 or cerulenin, respectively, were analyzed for SFFLRN-induced P-selectin expression as previously described.

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supernatants, although some APT1 was observed in pellets after centrifugation of cavitate from resting platelets, confirming by demonstration of increased P-selectin surface expression. Activation of platelets was induced by stimulation with SFLLRN, which stimulates platelets through protease-activated receptor-1 (Figure 1B). After activation, APT1 became more equally distributed between supernatant and pellets, demonstrating that some APT1 translocates to membrane or organized cytoskeletal structures after platelet activation. These results indicate that some, but not all, proteins involved in protein palmitoylation undergo activation-dependent translocation.

**APT1 Inhibits Platelet Activation**

The role of protein palmitoylation in platelet function is unknown. Nucleated cells overexpressing APT1 have previously been used to evaluate the role of protein palmitoylation in signal transduction. Because platelets are anucleate and therefore not amenable to standard genetic manipulation, we sought to increase the intracellular concentration of APT1 by permeabilizing platelets with SL-O in the presence of recombinant APT1. Permeabilization of platelets with SL-O enables the diffusion of proteins into platelet cytosol while maintaining their ability to activate in response to agonists. We first determined the effect of APT1 on total platelet palmitoylation in SL-O–permeabilized platelets. Incubation of SL-O–permeabilized, [3H]palmitate-labeled platelets with APT1 decreased platelet protein palmitoylation (Figure 2A). Quantitation of [3H]palmitate-labeled proteins using densitometry demonstrated that incubation with APT1 lead to a 48±4% (n = 3) decrease in total platelet protein palmitoylation. We next determined the effect of incubation with APT1 on P-selectin surface expression as a marker of platelet α-granule secretion. Incubation of intact platelets with APT1 had no effect on either basal or SFLLRN-induced P-selectin surface expression. In contrast, incubation of permeabilized platelets with APT1 inhibited SFLLRN-induced P-selectin expression by ≥90% (Figure 2B). APT1 inhibited SFLLRN-induced P-selectin expression in a dose-dependent manner with an IC50 of ~175 nmol/L (Figure 2C). These results show that depalmitoylation of platelet proteins by APT1 potently inhibits P-selectin expression, an established marker of platelet α-granule secretion.

**Protein Palmitoylation and Localization of Gq and SNAP-23**

Protease-activated receptor-1 couples to Gq to stimulate platelet granule secretion and aggregation. Gq is palmitoylated in platelets. To evaluate the effect of APT1 on Gq palmitoylation, platelets were labeled with [3H]palmitate and permeabilized with SL-O in the presence or absence of APT1. Gq was subsequently immunoprecipitated from platelet lysates and palmitoylation of Gq was evaluated by fluorography. Incubation of permeabilized platelets with APT1 resulted in a reduction of Gq [3H]palmitoylation to ~50% of basal levels as measured by densitometry (Figure 3A). Immunoblot analysis of Gq demonstrated that incubation with APT1 did not affect immunoprecipitation of Gq (data not shown). Thus, differences in the amount of palmitoylated Gq were not attributable to differences in protein loading. We next sought to determine the effect of removing palmitate on Gq expression. For these experiments, platelets were permeabilized with SL-O, washed, incubated in the presence or absence of APT1, and subsequently exposed to buffer, SFLLRN, or GTP-γ-S. Supernatants were collected after...
centrifugation of samples and assayed for G_{\text{m}}. Samples exposed to APT1 demonstrated increased G_{\text{m}} in supernatants compared with samples exposed to buffer alone (P<0.04). APT1 also increased G_{\text{m}} in supernatants of samples exposed to SFLLRN or GTP-\gamma-S. In contrast, neither SFLLRN nor GTP-\gamma-S had a statistically significant effect on loss of G_{\text{m}} into supernatants (Figure 3). Quantification of multiple experiments demonstrated that incubation with APT1 resulted in a 2- to 3-fold increase in the amount of G_{\text{m}} released in supernatants (Figure 3C). These data demonstrate that APT1 affects G_{\text{m}} membrane association.

SNAP-23 is a SNARE protein family member involved in platelet granule release.\(^{26}\) It associates with membranes via a membrane binding domain that contains 5 potential palmitoylation sites.\(^{15,27}\) We next sought to determine whether depalmitoylation of SNAP-23 affects its membrane association. SL-O-permeabilized platelets were incubated in the presence or absence of recombinant APT1 and subsequently exposed to buffer, SFLLRN, or GTP-\gamma-S. Supernatants were then evaluated for SNAP-23 by immunoblot analysis. SNAP-23 was barely detectable in the supernatants of samples not exposed to APT1 (Figure 4). In contrast, SNAP-23 was abundant in supernatants of samples incubated with APT1. Exposure to SFLLRN or GTP-\gamma-S did not affect translocation of SNAP-23 into supernatants. These data indicate that removal of palmitate from SNAP-23 results in its loss into the cytosol.

**Protein Palmitoylation and Platelet Function**

We next sought to determine whether inhibition of protein palmitoylation in nonpermeabilized platelets affected their activation. Cerulenin is a cell-permeable natural product that inhibits palmitoylation of H-ras--encoded and N-ras--encoded p21s and has been used to study protein palmitoylation in cell cultures.\(^{28-30}\) Incubation of platelets with cerulenin markedly inhibited protein palmitoylation (Figure 5A). We next tested the effect of cerulenin on platelet \(\alpha\)-granule secretion. Incubation of intact platelets with cerulenin inhibited SFLLRN-induced P-selectin exposure in a dose-dependent manner with an IC_{\text{50}} \approx 170 \mu\text{mol/L} (Figure 5B). Cerulenin also inhibited SFLLRN-induced platelet aggregation (Figure 5C). Protein palmitoylation has been invoked in signal transduction via many G-protein-coupled receptors as well as tyrosine kinase-coupled receptors.\(^{2-5,26,31-33}\) We therefore sought to determine whether cerulenin inhibits platelet activation induced by agonists that stimulate platelets through these 2 pathways. Cerulenin blocked platelet activation induced by physiologically relevant agonists that act through G-protein-coupled receptors including \(\alpha\)-thrombin, ADP, and epinephrine (Figure 5C). Cerulenin also inhibited platelet activation induced...
The observation that platelets incorporate \([^3H]\)palmitate into proteins indicates that protein palmitoylation occurs as a posttranslational modification in mature, resting platelets. Activation of platelets results in both increased and more rapid labeling of proteins with palmitate.\(^3^4\) Although the proteins responsible for these activities remain to be fully elucidated and characterized, we have identified the palmitoyl transfer proteins GODZ and HIP14, as well as the acyl-protein thioesterase APT1, in platelets. GODZ and HIP14 facilitate transfer of palmitate to free cysteines.\(^8^–10^\) These proteins occupy different subcellular compartments in resting platelets. GODZ is primarily cytosolic, whereas HIP14 is primarily bound to membrane and/or organized cytoskeletal structures (Figure 1). APT1 removes palmitate linked to proteins via thioester bonds. In the resting platelet, APT1 is primarily cytosolic. On activation, a significant portion of APT1 undergoes a translocation to a membrane and/or cytoskeletal compartment (Figure 1). These studies demonstrate responsiveness of the palmitoylation machinery to platelet stimulation. New palmitoyl transfer proteins continue to be identified.\(^5^–15^\) As tools and reagents become available, a more complete inventory of the platelet protein palmitoylation machinery will develop. The platelet may serve as a useful model in which to evaluate activation-dependent activities of palmitoyl transfer proteins and thioesterases.

The role of platelet protein palmitoylation in platelet function has not previously been assessed. Incubation of permeabilized platelets with APT1 reduces platelet protein palmitoylation and inhibits SFLLRN-induced platelet \(\alpha\)-granule secretion (Figure 2). Similarly, incubation of intact platelets with cerulenin decreases protein palmitoylation and inhibits agonist-induced \(\alpha\)-granule secretion and aggregation (Figure 5). These inhibitors act by distinct mechanisms. APT1 enzymatically removes palmitate from proteins, thus rapidly decreasing the amount of palmitate-associated with proteins in platelets. Cerulenin inhibits addition of new palmitic acid to proteins and, therefore, effectively prevents incorporation of \([^3H]\)palmitate into platelet proteins. These functional data show that the palmitoylation state of proteins is an important determinant of protease-activated receptor-1-mediated platelet activation.

Several functions have been ascribed to protein palmitoylation.\(^5^6^\) Perhaps the best-characterized function is to facilitate the association of proteins with membranes. We have found that removal of palmitate from 2 proteins involved in SFLLRN-induced platelet secretion, \(G_{\text{aq}}\) and SNAP-23, interferes with their membrane association (Figures 3 and 4). \(G_{\text{aq}}\) is coupled directly to protease-activated receptor-1. Palmitoylation of \(G_{\text{aq}}\) is thought to contribute to the cycling of this \(G_{\text{aq}}\) subunit between membrane and cytosolic compartments.\(^3^1\) Interference with \(G_{\text{aq}}\) localization likely contributes to the ability of APT1 to inhibit SFLLRN-induced platelet activation. These results are consistent with studies performed in nucleated cells transfected with \(G_{\text{aq}}\) subunits containing N-terminal cysteine residue mutations that prevent palmitoylation. Such \(G_{\text{aq}}\) mutants partitioned to cytosol.\(^3^2^,3^3\) SNAP-23 facilitates membrane fusion and acts at the most distal end of the cascade leading to platelet \(\alpha\)-granule secretion.\(^2^6\) A palmitoylated membrane-binding domain has been demonstrated to facilitate the membrane association of SNAP-25, a homologue of SNAP-23.\(^3^7\)

### Discussion

The observation that platelets incorporate \([^3H]\)palmitate into proteins indicates that protein palmitoylation occurs as a
Figure 5. Cerulenin inhibits agonist-induced platelet granule secretion and aggregation. A. [3H]palmitate-labeled platelets were incubated in the presence or absence of 500 μmol/L cerulenin, exposed to buffer or SFLLRN, and solubilized in sample buffer. [3H]palmitoylated platelet proteins were separated by SDS-PAGE and visualized by fluorography. B. Platelets were incubated with the indicated concentrations of cerulenin, exposed to either buffer or SFLLRN, and analyzed for P-selectin surface expression. Error bars represent the standard deviation of 4 experiments. C. Platelets were incubated with the indicated concentrations of cerulenin. Aggregation in response to 50 μmol/L SFLLRN, 0.1 U/mL α-thrombin, 10 μmol/L ADP, 10 μg/mL collagen-related peptide, 40 μg/mL collagen, or 10 μmol/L epinephrine (EPI) was subsequently assayed using standard platelet aggregometry.

demonstrated that protein–protein interactions dictate SNAP-25 membrane association. We find that incubation of SNAP-23 with APT1 results in loss of its association with platelet membranes (Figure 4). Gαq and SNAP-23 represent examples of how loss of palmitate reduces membrane-association of proteins involved in platelet function.

Results using an intravital mouse model demonstrate that interfering with platelet protein palmitoylation blocks platelet accumulation into thrombi. Cerulenin, an established inhibitor of protein palmitoylation, prevents palmitoylation of most platelet proteins (Figure 5A). To restrict cerulenin to platelets in our in vivo thrombus formation model, we performed a protocol using donor platelets incubated with cerulenin rather than infusing cerulenin directly into the mice. Cerulenin administered in this manner inhibited platelet incorporation into thrombi formed after laser-induced vascular injury by >60% (Figure 6). These studies suggest that protein palmitoylation may serve as a novel target for antithrombotic compounds. However, further in vivo investigations using large vessel occlusive thrombosis models will be required to substantiate this possibility. Our observations may further our understanding of the cardioprotective effects of omega-3 fatty acids, which are known to inhibit platelet function and which are established inhibitors of protein palmitoylation. Future
studies will identify novel targets to inform the development of compounds that block platelet protein palmitoylation and evaluate the effects of putative cardioprotective lipids on platelet protein palmitoylation.

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Disclosures
None.

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