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FUNCTIONAL ROLES FOR POST-TRANSLATIONAL MODIFICATIONS OF t-SNARES IN PLATELETS

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FUNCTIONAL ROLES FOR POST-TRANSLATIONAL MODIFICATIONS OF t-SNARES IN PLATELETS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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Lexington, KY

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Lexington, Kentucky

2016

ABSTRACT OF DISSERTATION

FUNCTIONAL ROLES FOR POST-TRANSLATIONAL MODIFICATIONS OF t-SNARES IN PLATELETS

Platelets affect vascular integrity by secreting a host of molecules that promote hemostasis and its *sequela*. Given its importance, it is critical to understand how platelet exocytosis is controlled. Post-translational modifications, such as phosphorylation and acylation, have been shown to affect signaling pathways and platelet function. In this dissertation, I focus on how these modifications affect the t-SNARE proteins, SNAP-23 and syntaxin-11, which are both required for platelet secretion. SNAP-23 is regulated by phosphorylation. Using a proteoliposome fusion assay, I demonstrate that purified IκB Kinase (IKK) phosphorylated SNAP-23, which increased the initial rates of SNARE-mediated liposome fusion. SNAP-23 mutants containing phosphomimetics showed enhanced initial fusion rates. These results, combined with previous work *in vivo*, confirm that SNAP-23 phosphorylation is involved in regulating membrane fusion, and that IKK-mediated signaling contributes to platelet exocytosis.

To address the role(s) of acylation, I sought to determine how syntaxin-11 and SNAP-23 are associated with plasma membrane. Using metabolic labeling, I showed that both proteins contain thioester-linked acyl groups which turn over in resting cells. Mass spectrometry mapping showed that syntaxin-11 is modified on C275, 279, 280, 282, 283 and 285, while SNAP-23 is modified on C79, 80, 83, 85, and 87. To probe the effects of acylation, I measured ADP/ATP release from platelets treated with the acyl-transferase inhibitor, cerulenin, or the thioesterase inhibitor, palmostatin B. Cerulenin pretreatment inhibited t-SNARE acylation and platelet function while palmostatin B had no effect. Interestingly, pretreatment with palmostatin B blocked the inhibitory effects of cerulenin suggesting that maintaining the acylation state of platelet proteins is important for their function. Thus my work indicates that the enzymes controlling protein acylation could be valuable targets for modulating platelet exocytosis *in vivo*.

KEYWORDS: Platelets, SNARE, Phosphorylation, Acylation, SNAP-23, Syntaxin-11

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April 7th, 2016

FUNCTIONAL ROLES FOR POST-TRANSLATIONAL MODIFICATIONS OF t-SNARES IN PLATELETS

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To Sheena, Raelene, Richard

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Chapter One: Introduction

Overview:

Platelets, as the smallest cells in the blood stream, are anucleate, discs that originate from megakaryocytes in bone marrow (1). Their structure is relatively simple. They contain granules, a membrane structure called the open canalicular system (OCS), microtubules, and mitochondria (2). When a vascular injury occurs, platelets use their numerous surface receptors to detect components, released and/or exposed, at the site of damage. They act as "first-aid kits" adhering to the injury, changing their shapes, and aggregating to form a hemostatic plug. The contents from platelet granules are also released upon activation. Cargo, released from platelets, control the vascular microenvironment at the injury site. Some cargo is important for recruiting other platelets, other cargo affects the surrounding cells. Due to these specific effects, it is important to understand what kind of molecules are in the granules, and how they are released.

Proteomic studies have identified hundreds of different molecules in platelet releasates (3,4). These components are stored in three distinct types of granules: α-granules, dense core granules, and secretory lysosomes. Cargo from α-granules contribute to primary and secondary hemostasis as well as to the *sequelae* of vascular injury (5). For instance, adhesive proteins, fibrinogen and von Willebrand Factor (VWF), play an important role in mediating platelet-platelet and platelet-endothelial interactions. Coagulation factors, such as Factors V, XI, and XIII, are also released

from α-granules. Dense core granules contain small molecules (*i.e.*, ADP, serotonin, polyphosphate, calcium), which are important for hemostasis (6). ADP is needed to recruit and activate more platelets and polyphosphates promote coagulation factor production (7). Secretory lysosomes contain a broad range of degradative enzymes, and though their precise function has not been established, they are thought to contribute to clot remodeling and to further platelet activation (7).

Platelets are also involved in inflammation and angiogenesis, and thus may contribute to cardiovascular disease, asthma, and cancer progression (8,9). Platelets release factors whose roles extend beyond initial hemostasis. Proangiogenic factors, such as vascular endothelium growth factor (VEGF) as well as antiangiogenic factors (angiostatin) have been detected in releasates (10,11). This contribution to angiogenesis may accelerate the progression of cancer diseases (12). Platelets also release a host of mitogens such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF), which are thought to promote wound healing (13). Finally, platelet can release several chemokines and cytokines which undoubtedly affect immune responses at the sites of vascular damage (14). Given this diversity of releasate components and their potential effects, it is critical that we understand how platelet secretion occurs and how it can be controlled.

Platelets are similar to other secretory cell types that release cargo from different classes of granules *i.e.,* mast cells, neutrophils, *etc.* In platelets, granules appear to undergo many of the steps that are thought to control regulated exocytosis, such as targeting, tethering, docking, priming. These steps culminate in a granule-plasma

membrane fusion event to release cargo. The secretion pathway is mediated by sequential protein-protein interactions leading up to the pairing of membrane associated SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment receptors) that span the two bilayers and promote membrane mixing and fusion. While many of these SNARE regulators are studied in our laboratory, this dissertation focuses on how post-translational modifications, specifically of the t-SNAREs, affects their roles in granule secretion.

Platelet Structure

Platelets are produced from megakaryocytes, and released into circulation (15,16). Their life span is around 7-10 days in human (3-5 days in mice). Because of their discoid shape and small size, platelets are carried along by the blood flow and pushed to the perimeter of the vessels, where they are better able to detect vascular lesions (1). In a resting platelet, its discoid shape is maintained by cytoskeleton, consisting of actin and tubulin. With observation from electron microscope (EM) images, the polymers of actin interconnect with spectrin strands, and form the spectrin-based membrane skeleton adjacent to cytoplasmic side of plasma membrane (17,18). In addition, actin filaments form a rigid network, which crosslinks with filamin and α-actinin (19). Filamin, as a scaffolding protein, can bind to GTPases, kinases, phosphatase and transmembrane proteins and localize them to plasma membrane in platelets (20). Also, each platelet contains one single microtubule, a polymer of αβ tubulin dimers, which forms a circumferential coil that is disassembled

upon platelet activation (21). In the surface of platelet disc, there are invaginations on plasma membrane, called the open canalicular system (OCS). OCS is a reservoir of membrane for activation-induced platelet spreading (22). The OCS, an invaginated tubular membrane region in platelets, is thought as site for granule fusion and as a membrane reservoir (23).

There are three distinct types of granules in platelets: dense core granules, α-granules, and secretory lysosomes. There are around 50-80 α-granules in per platelet and their size ranges from 200 to 500 nm (24). As the largest and the most abundant, α-granules contain the most diverse array of secretory molecules, such as, growth factors, adhesive receptors, and glycoprotein receptors. Some of the proteins are produced in megakaryocytes cells, such as platelet-specific proteins, platelet factor IV (PF4) and β thromboglobulin (5). α-granules can be identified by electron microscopy and immunofluorescence microscopy (5,25). Several established specific α-granule markers, such as P-selectin, vWF, PF4, and fibrinogen are used to identify α-granules. In this dissertation, PF4 was used as the α-granule marker in certain assays. P-selectin, on the luminal side of α-granule membranes, is exposed on the platelet surface and can be used as a marker to assay α-granule exocytosis by FACS. Many of the proteins discussed are synthesized *de novo* in the megakaryocyte; however, there are a number of proteins that are taken up by platelets and stored in α-granules. For instance, fibrinogen (Fg) is produce by the liver and taken up into α-granule (26). Interestingly, this platelet pool of Fg accounts for approximately 3% of the total circulating Fg. The second most abundant secretory granules are the dense

granules. These granules have smaller size and number (~7 per platelet) and they contain ADP, ATP, GDP, serotonin, Ca^{2+}, Mg^{2+} and other divalent cations (24). ADP and serotonin are activators in the "second wave" of platelet aggregation and are amplifiers of platelet activation. In the dissertation, ATP was used as a marker for dense core granule secretion. The secretory lysosomes represent the least abundant granules with about 5 per platelet. These granules contain membrane proteins such as LAMP-1, 2, and 3. Hydrolases, β-hexosaminidase, cathepsin, and acid phosphatases are also present in lysosomal granules (27). These enzymes are released only when platelets are stimulated with strong agonists (28). Overall, there are several hundred of molecules secreted from platelets, which affect vascular microenvironment. Platelets are involved in thrombosis, angiogenesis, inflammatory disease and cancer progression. So, it is essential to understand how to control platelet secretion.

SNARE Machinery in Platelets

Platelet granule secretion is the process mediated by protein-protein interactions. The targeting granules tether and dock to plasma membrane, where they fuse. Upon activation, intracellular [Ca^{2+}] increases and the secretory granules fuse with plasma membrane. This mediates the rapid release of granule cargo. At the same time, granule membranes fully fused to become part of the plasma membrane. SNARE proteins mediate these membrane fusion steps. SNARE proteins were initially described by Söllner *et al.*(29), who proposed that SNARE proteins are important for vesicle fusion in neurotransmitter release. There are numerous SNARE proteins identified in many species and this diversity suggests that specific SNAREs may be

responsible for distinct vesicles. In secretory cells, *e.g.* neurons, mast cells, and platelets, exocytosis is mediated by SNARE proteins residing on target membrane (t-SNAREs) and on vesicles/granules (v-SNAREs). t-SNAREs and v-SNAREs form a trans-bilayer, four-helix bundle of coiled-coil domains which is needed for membrane fusion (30).

In neuronal cells, syntaxin-1, SNAP-25 and VAMP-2 are the primary SNARE for synaptic release, and these three proteins form a four-helix bundle (2 helices from SNAP-25 and 1 each from VAMP-2 and syntaxin-1) (31). Weber *et al*. showed that fusion occurs when t-SNARE- and v-SNARE-containing proteoliposomes were mixed *in vitro.* These data showed that the SNAREs are the minimal element required for membrane fusion (32). The SNARE complex (VAMP-2, syntaxin-1 and SNAP-25) were shown to be SDS-resistant *in vitro* and in living cells, suggesting that once formed, it is very stable (33,34). The SNARE complexes may also contribute to other steps in the process, such as tethering or docking of vesicles to target membranes (30,35).

There are numerous SNAREs identified in platelets. t-SNARE isoforms include syntaxin-2, -4, -6, -7, -8, -11, -16, -17, -18, SNAP-23, SNAP-25 and SNAP-29; v-SNARE isoforms include VAMP-2, -3, -4, -5, -7 and -8 (36-38). Among those SNAREs, VAMP-8, syntaxin-11 and SNAP-23 are identified as primary SNAREs, which are required for membrane fusion in platelets. Analysis of syntaxin-2 and -4 double knockout mice showed that loss of syntaxin-2 and -4 does not affect platelet secretion. Syntaxin-11 has been shown to be required for platelet secretion in three

classes of granule secretion (39). As one of the primary t-SNAREs, syntaxin-11 is first reported as an atypical t-SNARE because it lacked a typical transmembrane domain and instead had a C-terminal cysteine-rich region (40). Syntaxin-11 is expressed in various tissues, but is enriched in cells from the immune system (41). It has been found on endosomes and was reported to participate in regulating late endosome to lysosome fusion in macrophages (42). Syntaxin-11, like syntaxin-2, -3, and -4, is also found on the plasma membrane in polarized Madin-Darby canine kidney (MDCK) cells (43). Although syntaxin-11 does not contain transmembrane domain (TMD) (40), it behaves as integral membrane protein (44). Syntaxin-11 mutations were identified in familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients (45), and syntaxin-11 is required for NK cell, $CD8^+$ T-cell degranulation and platelet secretion (46). Munc18-2 is syntaxin-11 chaperone and mutations in Munc18-2 disrupt its binding to syntaxin-11 resulting in decrease levels of syntaxin-11 (47). Ye *et al.*showed that syntaxin-11, but not syntaxin-2, or -4 is required for platelet secretion (39). In recent study, syntaxin-8 has been found to form complexes with syntaxin-11 in platelets and to be specifically important for dense core granule secretion and platelet aggregation but not essential for α- and lysosomal granule secretion (48).

The t-SNARE SNAP-23 is widely expressed in many tissues and and is highly expressed in platelets. Its homologues, SNAP-25 and SNAP-29 are expressed at much lower levels (37,38). SNAP-23 is widely believed to play a key role in exocytosis in most cells that do not express SNAP-25 (49). Due to its ubiquity, SNAP-23 deficiency

causes implantation embryonic lethality in mice (49). Anti-SNAP-23 antibody can inhibit granule docking to target membranes (50). SNAP-23 is important for exocytosis of platelet α-granules (51), dense granules (50) and lysosomal granules (52). SNAP-29 null platelets did not have secretion defect (53).

Platelet secretion is attenuated, but not ablated in VAMP-$8^{-/-}$ mice. VAMP-$3^{-/-}$, VAMP-$2^{+/-}$ and VAMP-$2/3^{-/-}$ platelets did not show significant secretion defects (54). These data suggested that VAMP-8 is the primary v-SNARE. Treatment of permeabilized VAMP-$8^{-/-}$ platelets with a VAMP-2/3 cleaving toxin ablated secretion, suggesting a secondary role for VAMP-2 and/or -3 (54). In our lab's recent studies, we showed that secretion of the three types of granule can be greatly decreased VAMP-$2/3/8^{-/-}$ platelets, further indicating that VAMP-2, -3, -8 (VAMP-8 as primary; -2, -3 as secondary) co-regulate platelet secretion in mouse platelets (Smita Joshi unpublished data). Consistently, there does appear to be a role for VAMP-7 in platelet exocytosis. Flaumenhaft and colleagues reported a partial dense core granule and α-granule secretion defect in VAMP-$7^{-/-}$ platelets (55). Taking all of these data into account, it would seem that VAMP-8 mediates to highest level of platelet secretion followed by VAMP-7 and that VAMP-2 or -3 can serve as compensatory VAMPs when neither VAMP-7 nor -8 is present.

SNARE Regulators in Platelets

While SNAREs alone are sufficient for membrane fusion (32), fusion is Ca^{2+}-independent and slow (min-hr) *in vitro*. *In vivo*, granule release is not only Ca^{2+}-dependent but also fast. It takes milliseconds for neurotransmitter release and

seconds for platelet exocytosis (56-58). SNARE proteins are core elements for membrane fusion. In secretory cells, regulators control both the temporal and spatial aspects of cargo release. Similar to neurotransmitter release, several SNARE effectors are Ca^{2+} sensors. Some are present in platelets. Munc13-4, Munc18-2, STXBP5/Tomosyn-1, as SNARE effectors, have received much attention because they interact with syntaxins (59-62). Deficiency in SNARE-regulators results in defective granule secretion (63,64).

Munc13-4

Munc13s contain two C2 (C2A and C2B) domains. Munc13s are thought to be priming factors in granule secretion. There are four isoforms detected in mammals: Munc13-1, Munc13-2, Munc13-3, and Munc13-4. Munc13-1, -2, -3 are exclusively expressed in brain. In a Munc13-1/2$^{-/-}$ mouse model, release from glutamatergic and from GABAergic neurons are completely blocked, indicating that Munc13-1 and Munc13-2 are required for neurotransmitter release. Mechanistically, Munc13-1 is thought to regulate neuronal secretion by promoting the transition of syntaxin-1 from its closed conformation to an open conformation that can form *trans*-SNARE complexes (65). Another homologue, Munc13-4 has been identified and is widely expressed in non-neuron tissues. Munc13-4 is a Rab 27-binding partner which participates in regulating Ca^{2+} triggered dense core granule secretion in platelets (66-68). Mutations in Munc13-4 are related to a fatal hyperinflammatory disorder called Familial Hemophagocytic Lymphohistiocytosis type 3 (FHL3). In the Munc13-4$^{-/-}$ mouse model, dense core granule secretion is abolished, and α- and

lysosomal granule secretions are attenuated (69). Munc13-4 contains two C2 domains (C2A and C2B) which are Ca^{2+} binding sites. *In vitro* fusion assays show that Munc13-4 regulates membrane fusion in Ca^{2+}- and phosphatidylserine (PS)-dependent manner and may span the two fusion bilayers to promote SNARE complex formation. (70).

Munc18-2

There are three isoforms in the Munc18 family, Munc18-1, -2, and -3, which are all expressed in platelets (71). Munc18-1 is mostly expressed in neuronal tissue and binds to syntaxin-1 and regulates SNARE complex assembly. Because Munc18-3 deficient mice did not survive past birth, platelets from Munc18-$3^{+/-}$ mice were used for granule secretion studies. There was no secretion defect detected (72). In Familial Hemophagocytic Lymphohistiocytosis type 5 (FHL5) patients, Munc18-2 is deficient. This causes a granule secretion defect in platelets, indicating that Munc18-2 is required for granule secretion in platelets (73,74) . Consistently, the expression of syntaxin-11 was also affected by the loss of Munc18-2.

Other Regulators

Rab27s is a member of the Rab family of small GTPases. Two isoforms, Rab27a and Rab27b have been detected in platelets. In a recent study, using a Rab27ab$^{-/-}$ mouse, Rab27s were shown to be required for dense core granule biogenesis and granule secretion, but not in α-granules and lysosomes, indicating Rab27s is important for granule packaging and secretion(75)

STXBP5/Tomosyn-1 was originally identified in rat brain as a syntaxin-1 binding protein, hence the name syntaxin binding protein 5 also called “friend of Syntaxin” or tomosyn (STXBP5//Tomosyn-1). All the isoforms of Tomosyn-1 (b-, m-, s-) contain a C-terminal of v-SNARE motif, suggesting that it helps form SNARE complex with syntaxin-1 and SNAP-25 (76). Tomosyn-1 is thought as a negative regulator of neuron transmitter release and vWF release from endothelial cells and this has been borne out in studies of knockout mice (77); however, it is a positive regulator that is required for granule secretion in platelets (78). Mice, deficient in the platelet STXBP5//Tomosyn-1 showed an enhanced bleeding diathesis, despite higher than normal levels of circulating vWF.

SNARE Phosphorylation

In addition to those regulators in platelets, post-translational modifications are ubiquitous in secretory cells and have been shown to be important for granule exocytosis. Phosphorylation is a post-translational modification and may affect protein-protein interaction, affinity of protein complex. Protein kinase C (PKC) was identified as one of the major kinases regulating protein phosphorylation. In response to phorbol ester, SNAP-25 phosphorylation was detected in many cell types, *e.g.*, PC12 cells, pancreatic β cells and chromaffin cells. Ser187 is one of the identified phosphorylation sites. PKA and casein kinase II (CKII) both can phosphorylate SNAP-25 *in vitro.* Interaction with SNAP-25 are disrupted when SNAP-25 was phosphorylated by PKA, but not CKII (79), indicating that SNAP-25 phosphorylation affects SNARE complex affinity. $I_{\kappa}B$ Kinase beta (IKKβ) directly phosphorylates

SNAP-23 at Ser95 and Ser120 in mast cells and in activated platelets. In another study, purified PKC can also phosphorylate SNAP-23, but not at Ser95 or Ser120. Based on data from our laboratory, it seems that IKKβ may be a downstream effector of PKC in activated platelets, since PKC inhibitors affect IKK activation but IKK inhibitors have no effect on PKC activation (80).

Another t-SNARE protein, syntaxin-4 is also phosphorylated in thrombin-activated human platelets, and its phosphorylation can be inhibited by PKC inhibitor (81). Munc18-1 is phosphorylated in response to phorbol ester treatment in adrenal chromaffin cells, and purified PKC can phosphorylate Munc18-1 at Ser313 *in vitro* (82). Phosphorylation of Munc18-1 by PKC reduced the binding affinity to syntaxin-1 in binding assay (82). Above all, it is indicated that Munc18-1 phosphorylation is important for granule secretion by PKC.

Phosphorylation of SNAP-23:

Phosphorylation of other SNAP-23 superfamily members has been detected. SNAP-25 is phosphorylated by PKC *in vitro* (83,84). SNAP-25 has been shown to be a PKC substrate in some cell types *e.g.*, PC12 cells (85,86), hippocampal organotypic cultures (87), pancreatic β cells (88) and adrenal chromaffin cells (89). Phosphorylation of SNAP-23 promotes its association with other SNAREs in the process of SNARE complex formation in mast cells (90). Phosphorylation of SNAP-23 enhances t-SNARE complex assembly by SNAK (SNARE kinase) (91). In activated mast cells and platelets, Ser95 and Ser120 are phosphorylated (83). In Figure 1-1, Ser95 and Ser120 are close to the cysteine-rich region between N- and C-

terminal coiled-coil domains. Upon activation, SNAP-23 phosphorylation appears to promote SNARE complex formation (syntaxin-11/SNAP-23/VAMP-8 and syntaxin-2/SNAP-23/VAMP-8). Consistently, SNARE complexes could not be detected in co-immunoprecipitation assay and SNAP-23 phosphorylation was also affected in IKKβ$^{-/-}$ platelet lysate (80). To understand the importance of phosphorylation in SNAP-23, it is critical to identify which kinase is responsible for SNAP-23 phosphorylation.

IKK Complex and its Inhibitors

The IKK complex is composed of catalytic and regulatory subunits. The catalytic subunits, IKKα and IKKβ, are regulated by IKKγ (92-95). All elements of IKK complex are present in platelets (80). Normally, IKK regulates NF-κB signaling pathway and thus several IKK inhibitors have been created to modulate inflammation and cell growth in cancer cells [32-34]. IKKβ inhibitors, such as BMS 345541, BAY 11-7082, and TPCA-1, have been used in preclinical trials as anti-cancer and anti-inflammatory agents (96-98). SNAP-23 is phosphorylated by IKKβ in mast cells (99). Recently, it was reported that IKK phosphorylates Ser95 and Ser120 of monomeric SNAP-23 as well as in SNARE complex in activated mast cells (83,99). This appear to promote SNAP-23 association with syntaxin-4 and VAMP-8 in lipid rafts (99). Phosphorylation of SNAP-23-IKK complexes was detected in lipopolysaccharide (LPS)-activated bone marrow–derived mast cells (100). IKKβ$^{-/-}$ platelets and inhibitor-treated platelets displayed decreased SNAP-23 phosphorylation, diminished SNARE complex formation, and decreased granule secretion (80) . Mice

treated with IKKβ inhibitors showed a significant increase in tail-bleeding time. These data show that IKKβ can phosphorylate SNAP-23 at Ser95 in platelets, which is important for platelet secretion. Both Ser95 and Ser120 are adjacent to the cysteine-rich region involved in anchoring SNAP-23 to membranes *via* its acylation (Figure 1-1) (101). It is unknown whether the phosphorylation of Ser95 and/or Ser120 is involved in regulation of SNARE complex assembly, selectivity, or membrane fusion. The role of phopho-Ser95 in regulating SNARE complex formation and membrane fusion remains uncharacterized. In the dissertation (Chapter 3), I will use *in vitro* fusion assay and mutagenesis to address some of these questions.

Acylation and Deacylation

In addition to phosphorylation, there are forms of post-translational modification (PTM), which are essential for protein function in platelets. Acylation has been identified in platelets, which is thought to be important in the regulation of platelet function (102,103). It is one of the PTM ubiquitously found in all eukaryotes. Unsaturated fatty acid, palmitate (C_{16}) reversibly forms lipid anchors for several target proteins *via* thioester bonds (S-palmitoylation) and irreversibly through amide bonds (N-palmitoylation). N-palmitoylation occurs at N-terminal cysteines or glycines and is especially found in secreted proteins (104). Due to the amide bonds, N-palmitoylation is an irreversible modification, similar to prenylation and myristoylation. As opposed to other PTMs, S-palmitoylation is readily reversible PTM and does not require any specific sequence motif. C_{16} covalently attaches to cysteine residues of proteins *via* a thioester bond. Different lengths of fatty acids, such as C_{18} (called stearate (18:0), C_{20}

(called arachidonate (20:4), attach to cysteine residues, are generally called S-acylation; however, S-palmitoylation is the most common among S-acylation.

S-acylation can affect its target protein's characteristic, and change its function, causing the effects in various events of the cells. Firstly, acylation, as one of the common PTMs, serves as membrane anchors, and also localizes the proteins into lipid rafts. Fatty acids covalently attached to proteins increase their hydrophobicity, and thus serve as membrane anchors especially for those proteins lacking TMD. For instance, SNAP-25, in neuronal and neuroendocrine cells, contains a cysteine-rich region, which is palmitoylated and mediates attachment of the t-SNARE to plasma membrane (105). For those proteins containing TMD, acylation may help proteins associate with the plasma membrane or redistribute to intracellular compartments, such as lipid rafts. Beyond that, acylation also plays an important role in cell signaling, protein localization, protein stability, and protein-protein interactions (106-109).

There are several hundred S-palmitoylated proteins in mammals (110,111). Dowal *et al.* analyzed palmitoylated proteins by proteomic approaches and revealed that ~215 proteins among ~1500 proteins are palmitoylated in platelets (112). Those 215 known or novel candidate, palmitoylated proteins have diverse functions such as: cell signaling, immune response, cell growth, apoptosis, metabolism, transport, protein folding, DNA repair. This suggests that acylation may play multiple roles in platelets.

Acylation of SNAREs

SNAREs acylation has been detected in diverse cells or tissues, such as neurons, neuroendocrine, mast cells and platelets. Platelet palmitoylation studies from Dowal

et al. showed that numerous SNAREs are acylated. SNAP-23, syntaxin-2, -8, -10, -11, -12 and VAMP-3, -4, -5, -7 were identified as known or novel candidates in platelet "palmitoylome" (112).

SNAP-25 is well-known acylated proteins, and its 4 cysteines in the linker region connecting the two α-helices domains can be all modified. In metabolic labelling experiment, [^{3}H] palmitic acid incorporated into SNAP-25 in CV-1 kidney cells, all the 4 cysteines can be palmitoylated (113). Acylation also appears to regulate intracellular trafficking and exocytic function of SNAP-25 (114). Besides SNAP-25, synaptic SNARE proteins, VAMP-2 (115) and syntaxin-1 (116), together with synaptotagmin I (113) in neurons were also reported, indicating that palmitoylation is important for SNARE protein-mediated membrane fusion in synaptic vesicle fusion.

The primary platelet t-SNAREs, SNAP-23 and syntaxin-11, lack TMDs and contain cysteine- rich regions (Figure 1-2). As the homologue of SNAP-25, SNAP-23 is also palmitoylated in HeLa cells (101) and platelets (112). SNAP-23 and its homologue SNAP-25 are thought to be associated with membranes by acylation and/or by association with syntaxin isoforms (117,118). There is a cysteine-rich region in the linker region between N- and C- terminus of SNAP-23 (Figure 1-2B), demonstrating that acylation of cysteine residues may change the hydrophobicity of SNAP-23 and thus cause it to behave like integral membrane proteins (119). SNAP-23 contains 5 cysteines in cysteine-rich region and one more cysteine compared with SNAP-25, and additionally, more SNAP-23 enriched into lipid rafts in PC12 cells. A single mutation of cysteines in SNAP-23 (similar to SNAP-25) causes the level of SNAP-23 associated with lipid rafts to drop to a level similar to that of SNAP-25, implying that cysteine number affect SNAP-23 distribution and association

in the plasma membrane (120) . Alternatively, acylation sites may affect membrane association, even lipid rafts association.

Although syntaxin-11 lacks TMD (Figure 1-2A), it behaves like an integral membrane protein in NK cells, HeLa cells and platelets (39,44). It is also shown that syntaxin-11 is acylated in natural killer cells and syntaxin-11 disassociated with plasma membrane when 5 cysteine residues were replaced with Ala at C-terminus (121). When C-terminus of syntaxin-11 was deleted, truncated syntaxin-11 in HeLa was still partially associated with membrane by immunofluorescence microscopy, indicating that the C-terminus are not determining factor for its membrane association in HeLa cells (44).

However, it is still unclear how acylation of t-SNAREs affect their localization and function of the proteins in platelets, and how acylation happens to t-SNARE membrane association and whether it can affect granule secretion in platelets or not. With those questions, biochemical assays and *ex vivo* experiments were performed to provide clues in the dissertation (Chapter 4).

Enzymes for Acylation Regulation

There are several hundred of acylated proteins identified in platelets. Where are the fatty acids coming from? The resource of palmitate is from palmityl-CoA at nanomolar concentrations (122); however, the cycle of acylation and deacylation of proteins changes dynamically. The localization of H-Ras and N-Ras can be regulated rapidly through reacylation/deacylation cycle. The half-life of N-Ras acylation is less than 20 min (123-126). How could the cycle be regulated rapidly within such low concentration of palmityl-CoA in physiological contexts? S-acylation is enzymatically

regulated by opposing activities of two families of enzymes: protein acyltransferases (PATs) and Acyl Protein Thioesterases (APTs). S-acylation is a reversible process *via* a thioester bond. The major role of PAT is to enzymatically attach palmitate to specific cysteine residues. Conversely, APTs catalyze the reaction to remove acyl group and thus depalmitoylate proteins. There are only four identified APTs in mammalian cells in Table 1-1: acyl protein thioesterase 1 (APT1), acyl protein thioesterase 2 (APT2), palmitoyl protein thioesterase 1 (PPT1), and palmitoyl protein thioesterase 2 (PPT2) (102,127). PPT1 and PPT2 were found in lysosomes and play an important role in degradation of palmitoylated proteins (128). Syntaxin-2/SNAP-23 complex can be released from platelets when treated with APT1 (specifically removing palmitate) and botulinum toxin C (specifically cleaving syntaxin isoform), indicating that APT1 also can regulate depalmitoylation of SNAP-23(129). APT2 is 68% homologous to APT1, but can specially depalmitoylate the axonal GAP43 (130).

Typical PAT contains a cysteine-rich domain and a conserved Asp-His-His-Cys (DHHC) motif (131-133). There are 24 genes encoding proteins called ZDHHC in mammals and 25 in human (134,135). PAT activities highly depend on DHHC domain, since mutations of the cysteine of DHHC abolish the activity of acylation and self-palmitoylation in ZDHHC proteins (136). The majority of PATs also contain two other conserved motifs, DPG (aspartate-proline-glycine) motif and TTxE (threonine-threonine-asparagine-glutamate) motif, and so far, little is known about their function (137) . Most of the PATs were detected in ER or Golgi; however, some

localized to plasma membrane (138). It is demonstrated that neural PATs exhibit distinct substrate specificity (139). For instance, HIP14 (ZDHHC17) enhances palmitoylation of SNAP-25 in neuronal cells (131,140). ZDHHC2 also palmitoylates SNAP-25 in PC12 cells (141). Nine ZDHHC genes related to PATs are identified and associated with human disease: Huntington Disease (HIP14/ZDHHC17) (142-144), Schizophrenia (ZDHHC8) (145), Alzheimer Disease (ZDHHC12) (146), Mental retardation (ZDHHC9/15) (147), and cancers (ZDHHC2/9/11) (148-151). So far, there are at least 13 ZDHHC proteins or their mRNAs detected in platelets (Table 1-1).

Overall, due to the reversibility of S-acylation, it can be regulated by opposing action of enzymes, PATs and APTs. In previous study, PATs harbor the specificity for acylating certain proteins and APTs are less specific, There are almost certainly more APTs unidentified. In the dissertation, there are some function studies to address the importance of acylation in platelet secretion. PAT and APT inhibitors were used in these studies.

Inhibitors of PATs and APTs

2-bromopalmitate (2BP) and cerulenin (2,3 epoxy-4-oxo-7,10 dodecadienamide**)** are widely used for regulating PAT enzyme activity (Figure 1-3) (154-156). Cerulenin is a natural and lipid-based inhibitor of fatty acid synthesis and protein palmitoylation (157). It can irreversibly alkylate cysteines in PATs to prevent acyl-enzyme adduct formation (158). Also, cerulenin, as an acylation inhibitor in platelets, inhibits platelet aggregation and thrombus formation *in vivo* (102). 2BP has been more commonly

used as an acylation inhibitor (132,159-161). This palmitate analog has been used in platelets and it inhibited acylation of CD63 and CD9, affecting the tetraspanin-tetraspanin interaction (159). In acylation studies, 2BP and cerulenin were used to treat washed platelets. One of drawbacks of PATs inhibitors is that 2BP and cerulenin are nonselective probes among the PATs.

Unlike the large numbers of PATs, only four APTs have been identified in the cells, which contain thioesterase signatures: both the S-H-D catalytic triad and a G-X-S-X-G motif. Increasing evidences show that only two cytosolic APT1 and APT2 are the major APTs responsible for deacylation in diverse substrates (162-164). For APT inhibitors, Palmostatin B is an inhibitor of β-lactone acyl protein thioesterase 1 as shown in Figure 1-4. In the presence of Palmostatin B, the proper localization of H-Ras and N-Ras was disrupted, and therefore the signaling pathway was down-regulated (165). Although Palmostatin B is a selective inhibitor for APTs, both isoforms, APT1 (IC_{50}=5.4 nM) and APT2 (IC_{50}=37.7 nM) are inhibited by Palmostatin B (166). Palmostatin B is used to study acylation of t-SNARE in my dissertation. Palmostatin M ((APT1 (IC_{50}=2.5 nM) and APT2 (IC_{50}=19.6 nM)), a more potent and more soluble analog, was further developed and tested as a deacylation inhibitor (167); however, it is not commercially available and could not be used for my studies. Both Palmostatin B and M are non-selective for APT1/2 isoforms as indicated by their IC_{50}.

Lipid Rafts in Platelets

Often, acylated proteins lacking a TMD, gain the ability to move around plasma

membrane, especially to lipid rafts (168). Lipid rafts are island-like subdomains of the plasma membrane, consisting of cholesterol, glycosphingolipids and raft-associated proteins (169). The lipid rafts can be regulated dynamically by cell signal pathway and palmitoylated proteins reside in the specific domain participate cell signaling events and protein complex formation. Acylated proteins have an affinity for lipid rafts; however, prenylated proteins have little affinity for lipid rafts (170), indicating that distribution of acylated proteins may change as raft changes its localization. For t-SNARE proteins in platelets, acylation as a lipid anchor may enhance proteins hydrophobicity. Syntaxin-11 and SNAP-23 redistribute into lipid rafts upon activation in platelets and also in mast cells (80,90). But it is unclear whether acylation will be one important factor for protein redistribution in lipid rafts or not. In the dissertation, lipid rafts were isolated for detecting t-SNARE redistribution upon platelet activation.

Dissertation Overview

Abnormal platelet function can cause bleeding or spurious thrombosis leading to strokes and heart attacks. Given its importance, it is critical to understand how platelet exocytosis is controlled. From previous study, it is believed that SNAREs form a four-helix bundle, and then mediate membrane fusion in platelets. Regulatory proteins, such as Munc13-4, Munc18-2, Tomosyn-1 and Rab 27, play an important role in regulation platelet granule secretion. At the same time, posttranslational modifications, such as phosphorylation and acylation, have been shown to affect signaling pathways and platelet function.

First, to identify the effect of phosphorylation in membrane fusion (in Chapter 3),

using an *in vitro,* proteoliposome fusion assay, we demonstrated that purified IκB Kinase (IKK) phosphorylates SNAP-23 and this increases the initial rates of SNARE-mediated liposome fusion. Both phosphorylation and fusion enhancement were blocked by the addition of the IKK inhibitors, BMS 345541 and TPCA-1. Phosphomimetic study showed that phosphomimetic group in Ser95 enhanced membrane fusion in proteoliposome fusion assay. These results, combined with previous work *in vivo,* suggest that SNAP-23 phosphorylation at Serine 95 is involved in regulating membrane fusion, and that IKK-mediated signaling contributes to platelet exocytosis.

As t-SNARE proteins, SNAP-23 and syntaxin-11, lack TMD, but are required for platelet secretion. Both of the t-SNAREs contain cysteine-rich region as putative acylation sites. Little is known about how SNAP-23 and syntaxin-11 behave as integral membrane proteins and whether acylation can affect SNARE mediated membrane fusion or not.

To answer these questions, biochemical and cell biological assays were performed in the first part (Chapter 4) of the dissertation. In the subcellular fractionation assays, syntaxin-11 and SNAP-23 are membrane associated proteins and redistributed into lipid rafts together with v-SNARE protein, VAMP-8, indicating that lipid rafts are the sites for membrane fusion. Using IP-ABE assays, we showed that both syntaxin-11 and SNAP-23 are modified by thioester-linked palmitic acid in platelets. To map the sites of acylation, syntaxin-11 and SNAP-23 were purified from platelets and analyzed by mass spectrometry. Our results showed that each cysteine in

cysteine-rich region can be acylated in syntaxin-11 and SNAP-23. [^{3}H]-palmitic acid metabolic labeling reveals that acylation of syntaxin-11 and SNAP-23 are dynamic, and turns over in resting platelets without affecting t-SNARE protein synthesis. To understand the potential role of changing acylation patterns, total acylation, aggregation, and ADP/ATP release were measured in platelets treated with an acyltransferase inhibitor, cerulenin, and/or acyl-protein thioesterase inhibitor, palmostatin B. Our data suggest that t-SNARE acylation turns over in resting platelets and that this cycling could be important for exocytosis. Overall, acylation of t-SNARE are dynamic and also can be regulated by APT and PAT inhibitors, and most importantly, acylation can affect SNARE mediated fusion in platelets.

In summary, SNARE protein modifications, *e.g.* SNAP-23 phosphorylation and SNAP-23/syntaxin-11 acylation, play key regulatory roles in platelet membrane fusion. The work in this dissertation points to two potential post-translation modifications that affect platelet t-SNARE function: acylation and phosphorylation. We have shown that targeting the enzyme responsible for these modifications can affect platelet function *ex vivo* and thus could be potential drug targets for controlling platelet exocytosis *in vivo*.

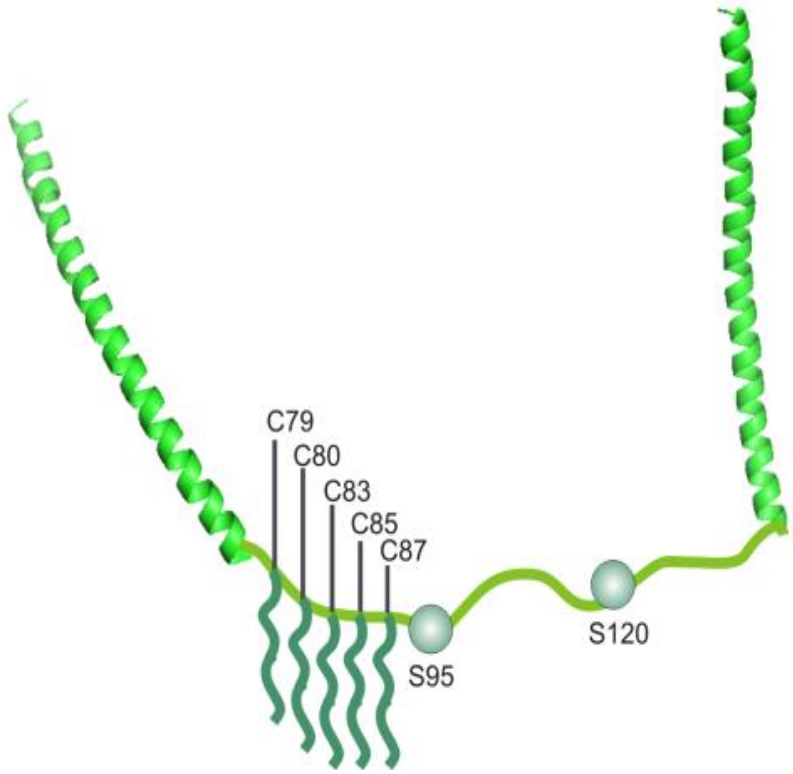


Figure 1-1 A Cartoon of SNAP-23, Highlighting Ser95 and Ser120

In emphasis on the importance of Ser95 and Ser120 phosphorylation, a cartoon of SNAP-23 highlighting Ser95 and Ser120, which locate close to the linker region, cysteine-rich residues, of N- and C-terminal coiled-coil regions. Acylation sites are indicated in the cysteine-rich residues of SNAP-23.

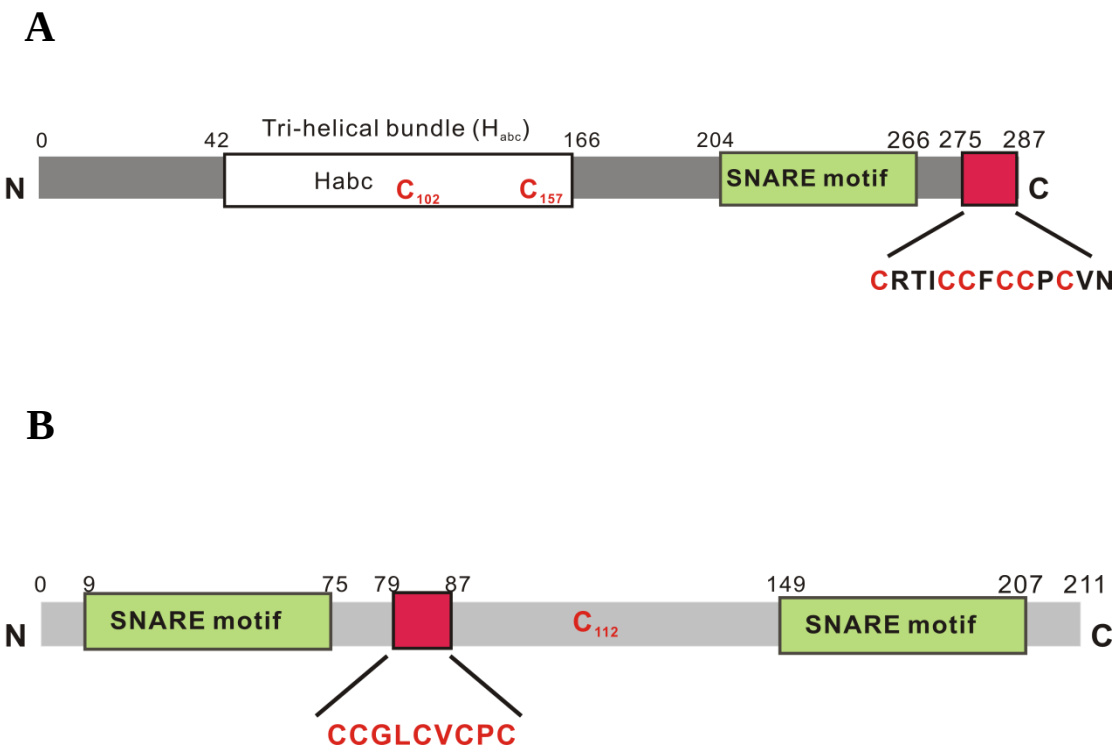
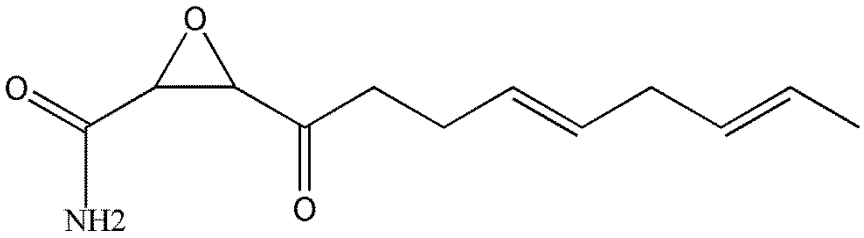
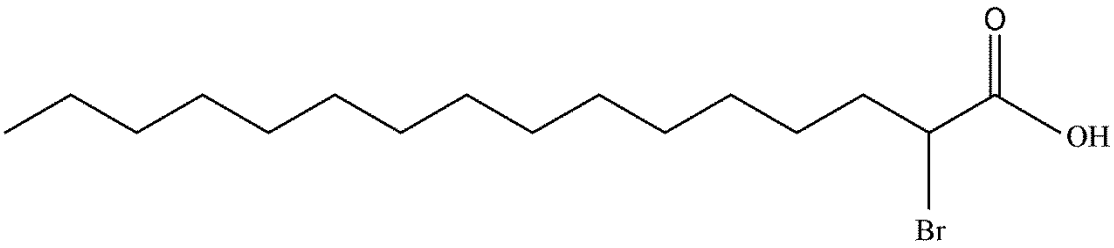


Figure 1-2 Schematic Diagrams of the Predicted Protein Domain Structures of Syntaxin-11 and SNAP-23

Predicted protein domain structures of syntaxin-11 (A) and SNAP-23 (B) based on the cDNA sequence from human syntaxin-11 and SNAP-23. A. The cysteine-rich region at C-terminus of human syntaxin-11 represents the acylation sites. There are two additional cysteines in helical bundle domain. B. There are highly conserved coiled-coil domains in human SNAP-23. Cysteine-rich region between coiled-coil domains represents the acylation sites.

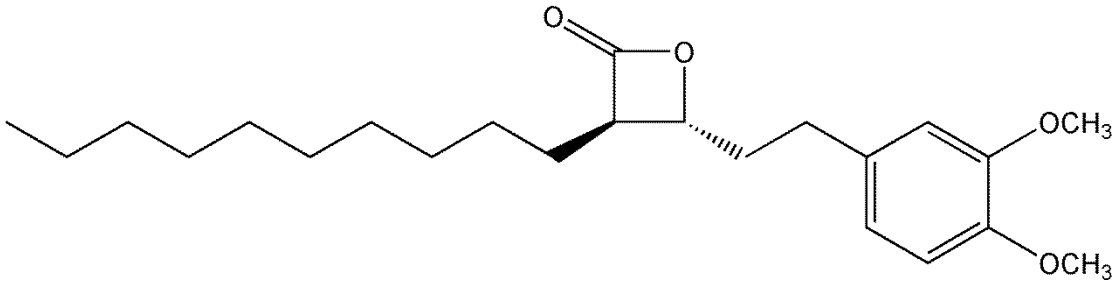


Cerulenin

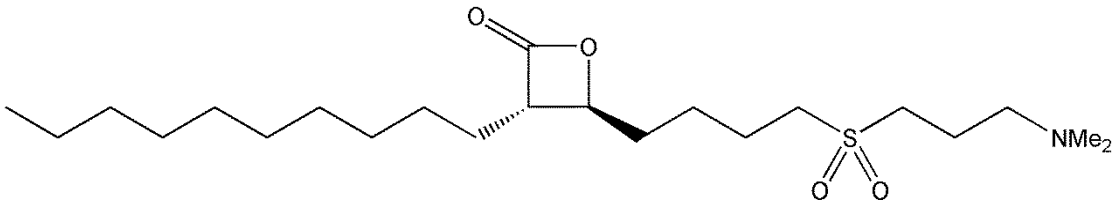


2-bromopalmitate (2BP)

Figure 1-3 Lipid-based Inhibitors of Acylation Applied in the Dissertation



Palmostatin B



Palmostatin M

Figure 1-4 Acyl Protein Thioesterae Inhibitors

Table 1-1 Palmitoyltransferases and Acyl-Protein Thioesterases Present in Platelets

Name	Protein in platelets	mRNA	Function
ZDHHC3	Yes(102)		S-palmitoyltransferase activity
ZDHHC17	Yes(102)		S-palmitoyltransferase activity
ZDHHC20	Yes(36) and (152)	Yes(153)	S-palmitoyltransferase activity
ZDHHC12		Yes	S-palmitoyltransferase activity
ZDHHC16		Yes	S-palmitoyltransferase activity
ZDHHC18		Yes	S-palmitoyltransferase activity
ZDHHC2		Yes	S-palmitoyltransferase activity
ZDHHC21		Yes	S-palmitoyltransferase activity
ZDHHC24		Yes	S-palmitoyltransferase activity
ZDHHC4		Yes	S-palmitoyltransferase activity
ZDHHC5	Yes (36,152)	Yes	S-palmitoyltransferase activity
ZDHHC7		Yes	S-palmitoyltransferase activity
ZDHHC8		Yes	S-palmitoyltransferase activity
APT1	Yes (102) (152) (127)		Hydrolyzes fatty acid
APT2	Yes (127)		Hydrolyzes fatty acid
PPT1;		Yes	Removes palmitate
PPT2		Yes	Removes palmitate

Chapter Two: Materials and Methods

Materials

Antibodies: Anti-syntaxin-11 rabbit polyclonal antibody (110113) was from Synaptic System GmbH (Gottingen, Germany). Anti-syntaxin-2, -4, and SNAP-23 polyclonal antibodies were generated in the Whiteheart laboratory as described previously (50,52). Anti-Munc18b antibody (sc-14563) was purchased from Santa Cruz Inc. (Santa Cruz, CA). Polyclonal rabbit-anti-human VAMP-8 antibody was generated in the Whiteheart laboratory using the cytoplasmic domain of human VAMP-8 (1-73 aa) as antigen; Anti-RabGDI polyclonal antibody was also generated in the Whiteheart laboratory by Dr. Tara Rutledge using recombinant RabGDIα as antigen. Fluorescein isothiocyanate (FITC)-conjugated CD62P (recognizes P-selectin was from BD Biosciences (San Jose, CA). Alkaline phosphatase conjugated secondary anti-murine, anti-rabbit, anti-sheep, and anti-goat IgGs were purchased from Sigma. Horseradish peroxidase (HRP) conjugated anti-rabbit IgG was from Sigma. HRP conjugated streptavidin was purchased from R&D systems.

General Reagents: Acid citrate dextrose (ACD) blood collection tubes (364606, BD Vacutainer®) were purchased from BD Diagnostics (Sparks, MD). Apyrase, cerulenin, diethylamine NONOate diethylammonium salt, urea and N-Ethylmaleimide (NEM) were purchased from Sigma (St Louis, MO). HisProbe™-HRP, BCA™ Protein Assay Kit, EZ-link®BMCC-biotin (1-Biotinamido-4-[4-(maleimidomethyl) cyclohexanecarboxamido] butane), hydroxylamine•HCl, iodoacetamide (IAA),

Pierce® streptavidin magnetic beads, and Pierce® ECL plus western blotting substrate were from Thermo Scientific (Rockford, IL). Complete, EDTA-free protease inhibitor cocktail was obtained from Roche (Indianapolis, IL). A23187, calcium ionophore, was from Calbiochem (San Diego, CA). APT1 inhibitor, palmostatin B was purchased from EMD Millipore (Billerica, MA). [9,10-^{3}H(N)]-palmitic acid was purchased from Perkin Elmer (Boston, MA, USA). Anti-Munc13-4 monoclonal antibody was generated using the domain C as antigen and given as a generous gift from Dr. Christian Wimmer (Basel Institute for Immunology, Basel, Switzerland). Recombinant human IKKβ, Amplex® red cholesterol assay kit, and Sypro®Ruby protein gel stain were from life technologies (Carlsbad, CA). QuikChange™ site-directed mutagenesis kit was from stratagene cloning systems (La Jolla, CA). β-octylglucoside was purchased from Research Products International Corp. (MT. Prospect, IL). PKCα was obtained from SignalChem (Richmond, BC, Canada). All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (PE), 1-palmitoyl-2-oleoyl phosphatidylcholine (PC), and 1,2-dioleoyl phosphatidylserine (PS), N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-PE) and N-(lissamine rhodamine B sulfonyl)-1,2- dipalmitoyl phosphatidylethanolamine (Rhodamine-PE), diacylglycerol (DAG). Amplex Red Cholesterol Assay Kit was purchased from Invitrogen, Life Technologies (Grand Island, NY). BMS-345541 and TPCA-1 were from Sigma-Aldrich

Methods

Mutagenesis in SNAP-23/Syntaxin-2 cDNA sequence: Constructs of syntaxin-2/SNAP-23 and VAMP-8 were designed and made by Dr. Michael C. Chicka in the Whiteheart laboratory (70). The detailed information is as below: DNA encoding the open reading frames of rat syntaxin-2 (amino acids 1-290; provided by J.M. Edwardson, University of Cambridge) and SNAP-23 (provided by E. R. Chapman, University of Wisconsin) was inserted into the pRSFDuet expression vector (Merck Millipore, Billerica, MA). All cDNAs were checked for variants and corrected to wild-type sequence if necessary. DNA encoding mouse VAMP8 (amino acids 1-101; provided by Wan Jin Hong, Institute of Molecular and Cell Biology, Singapore) was inserted into the pPROEX expression vector (Life Technologies, Grand Island, NY).

To replace the Ser95 residue of SNAP-23 with a negative charge amino acid, Asp or Glu in the construct of syntaxin-2/SNAP-23 by site-directed mutagenesis.

For the Asp mutation primers:

Forward primer sequence:

5’ ccaagaactttgag**gat**ggaaagaactataaggc 3’

Reverse primer sequence:

5’ gccttatagttctttcc**atc**ctcaaagttcttgg3’

For the Glu mutation primers:

Forward primer sequence

5’ ccaagaactttgag**gag**ggaaagaactataaggc 3’

Reverse primer sequence

5’ gccttatagttctttcc**ctc**ctcaaagttcttgg3’

For the Ala mutation primers:

Forward primer sequence

5’ ccaagaactttgag**gct**ggaaagaactataaggc 3’

Reverse primer sequence

5’ gccttatagttctttcc**agc**ctcaaagttcttgg3’

PCR conditions were as follows: 1 cycle at 95 ℃ for 30 sec, then 16 cycles at (95 ℃ for 30 sec, 57 ℃ for 1 min, 68 ℃ for 12 min) and 1 cycle at 68 ℃ for 12 min.

Amino acid substitutions were introduced using the site-directed mutagenesis (Stratagene). The sequence of clones was further confirmed by DNA sequencing at Davis Sequencing (Davis, CA).

Recombinant Protein Production: All syntaxin-containing complexes and VAMP-8 were overexpressed in the Rosetta Escherichia coli cells (Novagen) and target proteins with His_6 tag were purified from cell lysates by Ni^{2+}-NTA agarose chromatography.

For production of recombinant wildtype or mutated proteins (syntaxin-2/SNAP-23 and VAMP-8) , purified plasmid DNAs were transformed into the Rosetta Escherichia coli cells (Novagen) which harbor a plasmid (pRARE)

containing rare tRNA sequences (Novagen, Gibbstown, NJ). The colonies were amplified by culturing in 2,000 mL LB media containing 50 μg/mL kanamycin and 35 μg/mL chloroamphenicol (LB/kan/cam) overnight at 37°C, recovered by centrifugation, and inoculated into 8 L of SuperBroth in BioFlo 3000 fermentor (New Brunswick Scientific Co., INC., NY). Recombinant protein production was induced by adding 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hr at 37℃. The cells were harvested by centrifugation at 6,000 × g for 5 min and washed once in ice-cold PBS. The bacterial pellet was weighed and resuspended in breaking buffer. The cells were solubilized by 2% TritonX-100 for1 hr rotation at 4℃ and sonicated on ice. The lysate was subjected to ultracentrifugation at 100,000 × g for 1 hr. The supernatant was filtered using 0.2 μm nylon filter (Fisher Scientific) and applied to Ni^{2+}-NTA agarose chromatography. The purified recombinant proteins were eluted in 25 mM HEPES, pH7.4, 400 mM KCl, 500 mM imidazole, 10%w/v glycerol, 5 mM β-mercaptoethanol and 1% w/v β-octylglucoside at 4℃ and the aliquots were stored at -80℃.

Preparation of SNARE-Containing Proteoliposomes: All lipids were from Avanti Polar Lipids (Alabaster, AL). To reconstitute the v- or t-SNAREs into vesicles, phospholipids were mixed with protein in the presence of the detergent β-octylglucoside (32). After rapid dilution, the concentration of β-octylglucoside was below of the critical micellar concentration (CMC). Once the proteoliposomes were formed, the proteoliposomes were dialyzed overnight to get rid of detergent β-octylglucoside. And then the vesicles were concentrated by flotation in a density

gradient (Accudenze). For making t-SNARE proteoliposomes, t-SNAREs were reconstituted in 30% PE, 55% PC and 15% PS, and all phospholipids were added into glass tubes, which were soluble in chloroform. After mixing all the lipids, chloroform was then removed in N_2 evaporator (Parker Balston gas generator, Haverhill, MA) in the laboratory of Dr. Andrew Morris (University of Kentucky, Lexington, KY). The residual chloroform was removed by high vacuum for at least 1 hr. Then the phospholipids were resuspended by t-SNAREs, which were in protein elution buffer (25mM HEPES, 400 mM KCl, 500mM imidazole, 10% glycerol, 5 mM β-ME, 1% β-octylglucoside). The solution becomes clear because of the detergent. 2-fold volumes of dialysis buffer were added into tubes of vesicle, so that 0.33% (w/v) β-octylglucoside was below its CMC (0.73%). To obtain detergent-free membrane vesicle, the proteoliposomes were dialyzed in dialysis buffer (25 mM HEPES, 200 mM KCl, 10% Glycerol and 1mM dithiothreitol (DTT), pH 7.4). After dialysis, proteoliposomes will be concentrated using Accudenz gradient ultracentrifugation at 100, 000 × g at 4 ℃ for 4-6 hr, concentrated proteoliposomes will be collected from the 0/30% Accudenz interface.

v-SNAREs were reconstituted using a mix of 27% PE, 55% PC, 15% PS, 1.5% NBD-PE, (donor), and 1.5% Rhodamine-PE, (acceptor). All the steps were the same with t-SNARE proteolipsomes.

v-SNARE (VAMP-8) and t-SNARE (SNAP-23 + syntaxin-2) vesicles were reconstituted to give ~60 copies and ~95 copies per vesicle, respectively. Syntaxin-2 was a surrogate for syntaxin-11, as we cannot produce recombinant syntaxin-11 with

the appropriate acylation.

Fusion Assays: As shown in Figure 2-1, 10 μL t-SNARE vesicles were incubated with 0.5 mM ATP, 10 mM $MgCl_2$, and IKK (1.0 μg/reaction) at room temperature (RT). v-SNARE vesicles were incubated separately at RT. After 60 min, v- and half of the t-SNARE vesicles were mixed in 25 mM HEPES pH 7.4, 100 mM KCl, 1 mM DTT, and fusion was monitored at 37℃. Calcium (1 mM final) was added at t=20 min. The increase in NBD fluorescence was measured using a Bio-TEK FLx800 Microplate Fluorescence Reader and KC4 software with data acquisition every 1.5 min. After 60 min, 15 μL of 5% n-dodecyl-β-D-octylglucoside was added to obtain the maximum fluorescence. Fusion was plotted as the percent of maximum fluorescence over time. Some aliquot of t-SNAREs were analyzed by western blotting using anti-phospho-Ser95 and anti-SNAP-23 antibodies. For inhibitor study, 5 μM BMS-345541 or 0.5 μM TPCA-1 were added to the t-SNARE mixture containing 1.0 μg IKK and fusion was monitored after 60 min.

Mouse Platelet Preparation: Mice were euthanatized by CO_2 inhalation. After thoracic region were exposed, blood was collected from a ventricle by 1 mL syringe filled with 100 μL 3.8% sodium citrate and then mixed with PBS (pH 7.4) by 1:1 ratio, and incubated with prostaglandin I_2 (PGI_2) (10 ng/mL; 5min), followed by centrifugation at 237 $\times g$ for 10 min at room temperature (RT). Platelet-rich plasma (PRP) was carefully recovered avoiding disrupting red blood cell layer. Platelets were pelleted at 483 $\times g$ for 10 min at RT. The pellets were resuspended in HEPES/Tyrode buffer (20 mM HEPES/KOH, pH 6.5, 128 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 0.4

mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM D-glucose) supplemented with 1 mM EGTA, 0.37 U/mL apyrase, and 100 ng/mL prostaglandin I_2 (PGI_2) (Ann Arbor, MI). Platelets were washed and resuspended in HEPES/Tyrode buffer (pH 7.4) without EGTA, apyrase, or PGI_2. Platelets were counted with a Z2 coulter particle count and size analyzer (Beckman/Coulter, Fullerton, CA) and adjusted to 1~2 $\times 10^9$/mL for further use.

Human Platelet Preparation: Fresh human platelets from healthy donors were isolated and adjusted to 1 $\times 10^9$/mL. Blood was collected into acid citrate dextrose (ACD) collection tubes at UK clinic. After collection, all the blood were transferred into 50mL conical tubes and centrifuged at 250 $\times$ g for 20 min at RT to separate red blood cells from platelet rich plasma (PRP). The PRP was carefully transferred to a 15 mL tube containing 0.37 units/mL apyrase, 100 ng/mL PGI_2 for 10 min at RT, and then centrifuged at 900 $\times$ g for 10 min. The pellets, platelets, were resuspended with HEPES-Tyrode's buffer pH 6.5 containing 0.37 units/mL apyrase, 100 ng/mL PGI_2 for 10 min at RT. Finally, washed platelets were obtained by centrifugation at 850 $\times$ g for 8 min and resuspended with HEPES-Tyrode's buffer pH 7.4. The concentration of platelets was measured by a Z2 coulter particle counter (Beckman/Coulter, Fullerton, CA) and adjusted to the indicated concentrations.).

Subcellular Fractionation of Platelets: Subcellular fractionations were performed by following the Whiteheart laboratory protocol (50). Washed human platelets (1 $\times 10^9$/mL) were resuspended in HEPES/Tyrode buffer (pH 7.4) with protease inhibitors, and were disrupted by 5 freeze-thaw cycles to break plasma membrane. Plasma

membrane protein was spun at 100,000 × g for 1 hr at 4℃. The supernatant, cytosol protein, was kept, and plasma membrane was treated with 1% TritonX-100 and kept on ice for 30 min. After TritonX-100 treatment, samples were subjected to 100,000 × g centrifugation for isolating detergent soluble and insoluble fraction. The subcellular fractionations were subjected to SDS-PAGE and western blotting analysis.

Platelet Aggregometry: Washed platelets (500 μL, 4 × 10^8/mL) were warmed and stirred by a metal stirring bar with 800 rpm at 37℃ in a siliconized glass cuvette (Chrono-log, Havertown, PA) as shown in Figure 2-2. The assay was carried out in Model 460Vs Lumi-Dual aggregometer (Chrono-log). Agonists were added into the washed platelets, and the data were collected via a Model 810 Aggro/Link computer interface and Aggro/Link software (Chrono-log).

Platelet ATP Release: ATP release from dense core granules were carried out using aggregometer. Washed platelets (475 μL) were preincubated with Chrono-Lume® reagent (Chrono-log) containing luciferin and luciferase for 2 min at 37℃ under stirring condition at 800 rpm. The agonists were added into the reaction to initiate ATP release upon platelet activation. The product of luciferin by ATP-driven luciferase, was monitored by absorption at 560 nm using the same interface and software as platelet aggregation assay.

Metabolic Labeling With [^{3}H]-Palmitate in Platelets: For metabolic labeling, washed platelets were incubated with 200 μCi/mL [9,10-^{3}H(N)]-palmitic acid for 1 or 2 hr at 37℃, in HEPES-Tyrode's buffer, pH 7.4 in presence of 1% fatty acid-free bovine serum albumin (BSA). For other experiments, platelets were from human PRP

obtained from the Kentucky Blood Center as units (Lexington, KY). The platelet isolation from PRP steps were the same as fresh platelet isolation as described in Human Platelet Preparation.

Platelet [Ca^{2+}] Measurements using Fura-2AM: Intraplatelet calcium was measured using Fura-2-acetoxymethyl ester (Fura-2AM) and experiment procedure was modified from the Whiteheart laboratory protocol- Ren et al. (54). Washed platelets (4×10^8/mL) in HEPES-Tyrode's buffer (pH7.4) were incubated in presence or absence of cerulenin at 37 ℃ for 1 hr. Then 1 μM Fura-2AM (Invitrogen) was added into platelets at 37 ℃ for 1 hr. After incubation, the Fura-2 loaded platelets were resuspended in HEPES-Tyrode's buffer (pH7.4). The platelet concentration was adjusted to 2×10^8/mL. 0.7 mM $CaCl_2$ and 750 μL platelets were added to siliconized cuvettes and stimulated with 0.1 U/mL thrombin and constant stirring. Fluorescence was analyzed by excitation at 340 nm and 380 nm, and emission was measured at 509 nm using a model LS55 Luminescence Spectrometer (Perkin-Elmer Cetus). The ratio of emissions was calculated simultaneously using FL WinLab4.0 software (Perkin-Elmer Cetus) and used to calculate free calcium levels.

Cholesterol Depletion of Platelet: For cholesterol depletion, washed platelets were incubated with, a cholesterol depletion reagent, 10 mM methyl-β-cyclodextrin (MβCD) at 37°C for 30 min. After incubation, platelets were washed and resuspended at 4×10^8/mL in HEPES-Tyrode's buffer (pH7.4). The pretreated platelets were used for aggregation by agonists and ATP release assay. Half parts of platelets were lysed

in lysis buffer containing 1% TritonX-100, and subjected to sucrose-gradient centrifugation for lipid raft isolation.

Cholesterol Assay: Washed mouse platelets (5×10^8) or washed human platelets (1×10^8) were resuspended in in HEPES-Tyrodes Buffer (pH 7.4). Lipids were extracted with 400 μL chloroform: isopropanol:NP-40 (7:10:0.1) by vortexing and sonication, and then the extracts were centrifuged at 15, 000 × g at RT. The liquid was transferred to a new tube, and dried to remove organic solvent. The cholesterol assay was set up as follow: the reagents from Amplex Red Cholesterol Assay Kit (Life technologies). The dried lipids were dissolved in 1X Reaction Buffer by sonication and vortex mixing. Cholesterol reference standard was diluted to produce cholesterol concentration curve (0 to 8 μg/mL). In the reaction, it contained 300 μM Amplex Red reagent containing 2 U/mL HRP, 2 U/mL cholesterol oxidase and 0.2 U/mL cholesterol esterase. Once diluted samples were added into reaction, it took 30 mins for incubation at 37 ℃. Fluorescence was measured in reader at excitation of 544 nm and emission at 590 nm.

Platelet Lysis and Sucrose-Density-Gradient Fractionation: Washed platelets (1×10^9) was lysed with 2× lysis buffer (50 mM MES (pH 6.5), 300 mM NaCl, 2% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), protease inhibitor cocktail, and 4 mM Na_3VO_4) and mixed with 80% sucrose to make 40% sucrose-lysate mixture. The mixture was laid into ultracentrifuge tube (12 mL; 14 mm × 89 mm Beckman), and then 30% sucrose, 5% sucrose were laid sequentially on the top. The centrifuge tubes were applied on the rotor and centrifuged at 200,000 × g at

4 ℃. After centrifugation, the samples were collected in 1mL/ fraction by peristaltic pump (RAININ Instrument Inc.). Each fraction was concentrated by tricholoracetic acid (TCA) precipitation and subjected to western blotting.

Immunoprecipitation: Washed unlabeled or [^{3}H]-labeled platelets (500 μl) were warmed at 37 ℃ for 5 min and thrombin was added to activate platelets in stimulated group. 1 × 10^9 platelets were incubated in presence or absence of inhibitor and activated with thrombin (0.1 U/mL) , and then lysed with 2× lysis buffer (40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2% Triton X-100, 2% sodium deoxycholate, 5 mM sodium pyrophosphate, 2 mM Na_3VO_4, and protease inhibitor cocktail). The lysates were kept in ice and clarified by centrifugation with 13,000 RPM, for 5 min and the supernatants were incubated with rabbit IgG and Protein A-Sepharose 4 (PAS) Fast Flow (GE Healthcare, Piscataway, NJ) for pre-clear step and the PAS beads were discarded. And then antibodies of syntaxin-11 or SNAP-23 were incubated with platelet lysates for 2 hr at 4 ℃, and after 3 time wash, PAS beads were added into platelet lysates for 1 hr rotation at 4 ℃, and then 3 time wash. Proteins were eluted with 2×SDS loading buffer for 8 min at 95 ℃. For [^{3}H]-labeled platelets, Proteins were eluted with 2×SDS loading buffer (no β-mercaptoethanol or DTT) for 8 min at 95 ℃. The immunoprecipitates were analyzed by SDS-PAGE and western blotting.

Immunoprecipitation (IP)-Acyl-Biotinyl-Exchange (ABE) Experiments: IP and ABE experiments were performed for detecting acylation of t-SNARE proteins as shown in Figure 2-3, and protocol are modified from Brigidi *et al.*(171). For cerulenin

or palmostatin B treated experiments, washed platelets (1×10^9/mL) were incubated in presence or absence of drugs for 2 hr, and then washed and resuspended in HEPES/Tyrode buffer (pH 7.4). After treatment, platelets were lysed with ice cold 1x Lysis Buffer (with protease inhibitors, 1 mM PMSF, 2 mM Na_2VO_4, 2% Triton X-100, 2% sodium deoxycholate, 50 mM Tris-HCl pH7.5, 150 mM NaCl) containing 50 mM NEM. The Eppendorf tubes were rotated at 4℃ for 1hr. Anti-syntaxin-11 and anti-SNAP-23 antibodies were added into lysates and incubated at 4℃ for overnight. After overnight-incubation, PAS was pelleted by centrifugation at 500 × g for 1min and then washed three times with 1x Lysis Buffer containing protease inhibitors to get rid of NEM. IP samples with PAS beads were treated with 0.1M Hydroxylamine (HA) containing Lysis Buffer (pH7.4) for 30 min rotation, which removes palmitate from cysteines, and ready for biotinylation with BMCC-biotin. At the same time, the omission of HA cleavage was used as a negative control. After cleavage step, IP samples with PAS beads were incubated with 1.5 μM BMCC-biotin for 1 hr at 4℃ to for biotinylation formation. To get rid of unreacted BMCC-biotin, PAS beads were resuspended in Lysis Buffer, and proteins were eluted by 2× SDS-PAGE-Loading buffer, and then samples were boiled at 95℃ for 8 min.

Human Platelet Fluorescence-Activated Cell Sorting (FACS) Analysis: Washed human platelets were pre-treated with cerulenin as these concentration: 0 μM (as control group), 56 μM, 167 μM and 500 μM and incubated at 37℃ for 2 hr. 1 mM Ca^{2+} was added into platelets and incubated for 30 min prior to stimulation of platelets by agonists. 0.1 U/mL thrombin or 4 μM A23187 was used to stimulate platelets for 3

min, which were stopped by hirudin, and then FITC anti-human CD62P, P-selectin was added into platelets respectively for 10 min incubation. Then transfer samples to FACS tubes and ready for analysis by flow cytometry.

In the depletion of cholesterol study, washed platelets were pre-treated with 10 mM methyl-β-cyclodextrin (MβCD) for 15 min at 37℃, and then FACS were carried on with MβCD treated platelets.

Purified t-SNARE Preparation for Mass Spectrometry Analysis: Syntaxin-11 and SNAP-23 from washed platelets were purified by IP experiments and modified by IAA after HA cleavage. After trypsin digestion, peptide fragments from the two t-SNAREs were fractionated by HPLC and fragmentation in a TOF/TOF spectrometer used to identify acylation sites as indicated in Figure 2-4

Liquid Chromatography-electrospray Ionization-tandem Mass Spectrometry (LC-ESI-MS/MS) Analysis: All mass spectra reported in this study were acquired by the University of Kentucky Proteomics Core Facility. Syntaxin-11 and SNAP-23 from washed platelets were purified by IP experiments and modified by IAA after HA cleavage. After trypsin digestion, tryptic peptides were extracted, concentrated and injected for nano-LC-MS/MS analysis as previously described in Yang *et al.*(172).

LC-MS/MS analysis was performed using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Eksigent Nanoflex cHiPLC™ system (Eksigent , Dublin, CA) through a nano-electrospray ionization source. The peptide samples were separated with a reversed phase cHiPLC column (75 μm x 150 mm) at a flow rate of 300 nL/min. Mobile phase A was water with 0.1%

(*v*/*v*) formic acid while B was acetonitrile with 0.1% (*v*/*v*) formic acid. A 50 min gradient condition was applied: initial 3% mobile phase B was increased linearly to 40% in 24 min and further to 85% and 95% for 5 min each before it was decreased to 3% and re-equilibrated. The mass analysis method consisted of one segment with eight scan events. The 1st scan event was an Orbitrap MS scan (300-1800 m/z) with 60,000 resolution for parent ions followed by data dependent MS/MS for fragmentation of the 7 most intense ions with collision induced dissociation (CID) method.

MS/MS Protein Identification: The LC-MS/MS data were submitted to a local mascot server for MS/MS protein identification *via* Proteome Discoverer (version 1.3, Thermo Fisher Scientific, Waltham, MA) against a customized database containing syntaxin-11 or SNAP-23. Typical parameters used in the MASCOT MS/MS ion search were: trypsin digestion with maximum of two miscleavages, cysteine carbamidomethylation, cysteine nethylmaleimide modification, methionine oxidation, a maximum of 10 ppm MS error tolerance, and a maximum of 0.8 Da MS/MS error tolerance. A decoy database was built and searched. Filter settings that determine false discovery rates (FDR) were used to distribute the confidence indicators for the peptide matches. Peptide matches that pass the filter associated with the strict FDR (with target setting of 0.01) were assigned as high confidence. For MS/MS ion search, proteins with two or more high confidence peptides were considered unambiguous identifications without manual inspection. Proteins identified with one high confidence peptide were manually inspected and confirmed.

Anti-syntaxin-11 Antibody Purification: For affinity purification of the anti-syntaxin-11 antibody; purified syntaxin-11 was coupled to 2 mL CNBr-activated Sepharose beads for overnight incubation at 4℃. The column was washed with 20 volumes of PBS to remove unbound protein. 10 mL of 100 mM ethanolamine (pH 8.0) was added and incubated for 6 hr at 4℃ with gently rocking to block any residual reactive groups left on the column. After washing the column with 10 volumes of PBS, the antibody purifying column was ready to be used. Five milliliters of sera were diluted, 1:1, with TBS and filtered through a 0.2 μm filter. The filtered sera was loaded onto the column and incubated overnight at 4℃. To wash out the unbound antibody, the column was extensively washed with TTBS containing1% Tween-20 until the A280 was less than 0.02. The column was eluted with 10 volumes of Elution Buffer. One milliliter fractions were collected into 1.5 mL microtubes containing 50 μL the Neutralizing Buffer (1M Tris, pH 8.0). Antibody was collected, concentrated, dialyzed against PBS, and stored as aliquots at -20℃. The affinity-purified anti-syntaxin-11 antibody was used for immunoprecipitation of endogenous syntaxin-11 from platelet extracts in the dissertation.

Triton X-114 Phase Separation: Triton X-114 is the nonionic detergent solution, and can be homogeneous at 0℃, but separated into an aqueous phase and a detergent phase above 20℃ (173). Hydrophobic proteins will go into Triton X-114, the detergent phase; at the same time, hydrophilic proteins will be in aqueous phase. Triton X-114 phase separation was designed for platelets membrane protein separation, Platelet membrane proteins can be lysed in Triton X-114, hydrophilic

proteins were found in the aqueous phase, whereas hydrophobic proteins, such as integral proteins or lipid-anchored proteins were recovered in the detergent phase. Experiment procedure was modified from Kutzleb *et al*. (173).

Platelet membrane pellet (1×10^9 platelets) were lysed in 2% Triton X-114 and treated with or without 0.3 M HA at 4 ℃ for 1 hr. The lysis of membrane pellets was transferred to a microcentrifuge tube and then subjected to centrifugation for 15 min at 10,000 × g at 4 ℃ to get rid of insoluble fraction. Supernatant was taken out in to a fresh microcentrifuge tube and incubated at 37 ℃ for 3 min for phase partitioning and then subjected to centrifugation for 1 min at 10,000 × g at RT. The upper (aqueous) phase and the lower (detergent) phase were separated. Re-extractions of aqueous and detergent phase were done for additional twice for further purification. Extracted samples were precipitated by TCA to get rid of Triton X-114, were subjected for SDS-PAGE gel and then probed for analysis with anti-syntaxin-11, anti-SNAP-23, anti-VAMP-8, and anti-syntaxin-4 antibodies.

Statistics Analysis: Data acquired in the dissertation were analyzed and performed using GraphPad Prism 6. Two-way ANOVA or Student's t-tests were performed. P values < 0.05 were considered significant.

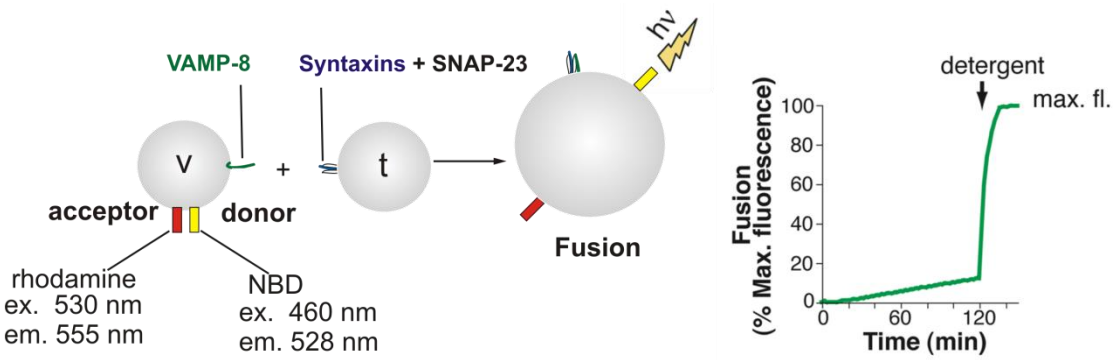


Figure 2-1 Proteoliposome Fusion Assay

The in vitro fusion assay is illustrated. Labeled v-SNARE (VAMP-8) proteoliposomes with Förster resonance energy transfer (FRET) pair (NBD and rhodamine, as a donor and acceptor respectively) were mixed with unlabeled t-SNARE (syntaxin-2/SNAP-23) liposomes. During the membrane fusion happening, the distance between NBD and rhodamine was increased and thus more NBD signal was recorded. Fusion data were normalized to the maximum donor fluorescence as described in Methods.

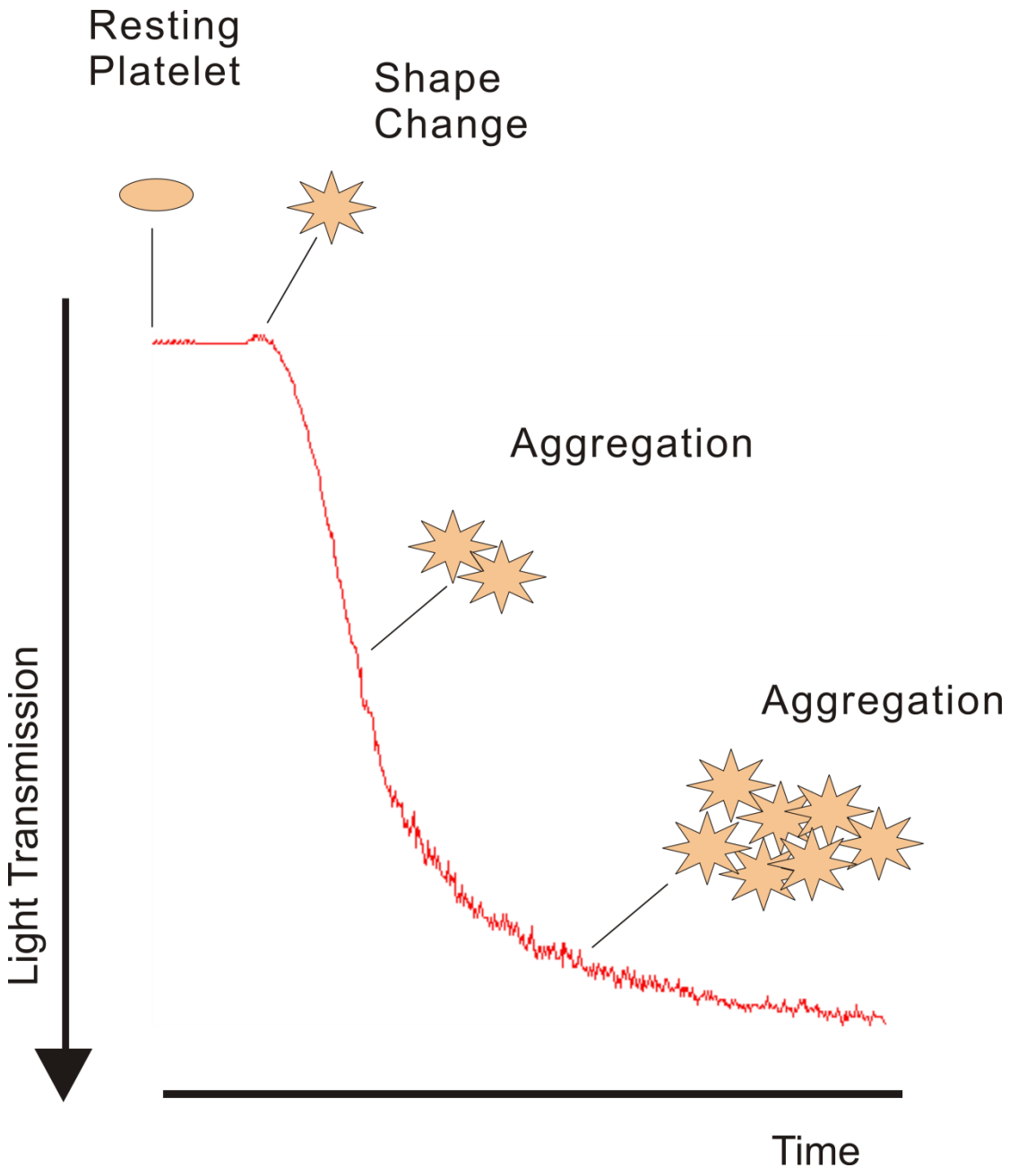
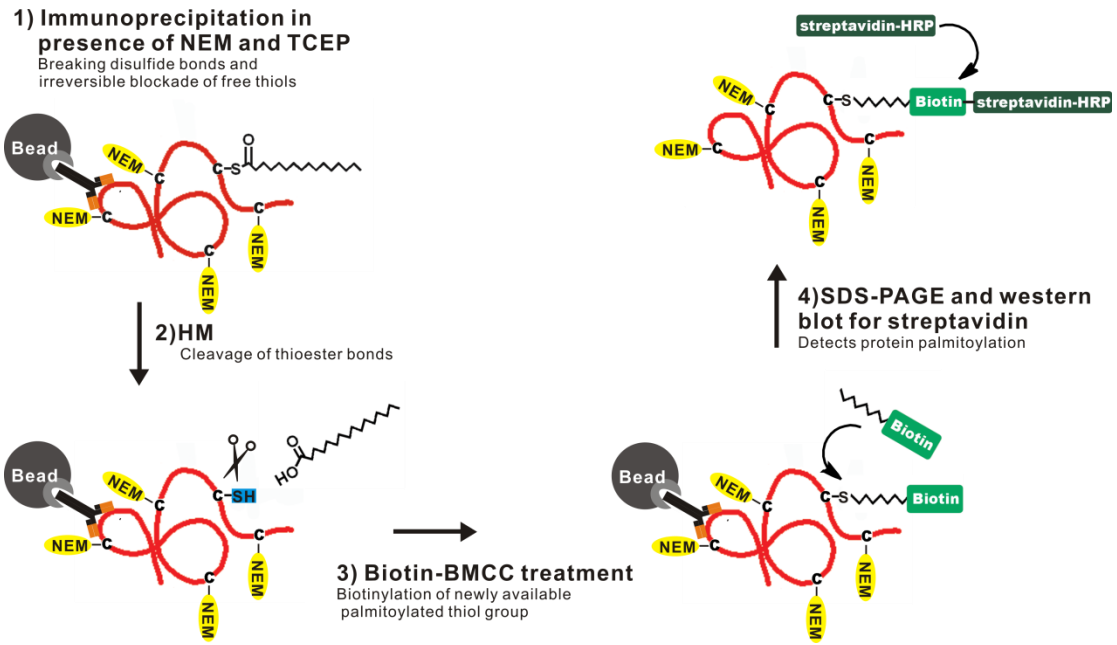

Resting
Platelet
Shape
Change
Aggregation
Aggregation
Light Transmission
Time

Figure 2-2 Schematic of Platelet Aggregation Detection by Aggregometry

In the aggregometer, washed platelets (4×10^8/mL) are added and stirred in a cuvette at 37℃ until a steady baseline is achieved. When an agonist is added, the response recorded that the platelets aggregate and absorb less light. So the transmission increases and this is detected by the photocell. The aggregometer is calibrated by: a cuvette containing washed platelets which equates to 0% light transmission. A second cuvette containing dH_2O is set to 100% light transmission. Upon platelets stimulated by agonists, the aggregation curve will be recorded by aggregometry.



Revised from *J Vis Exp. 2013 Feb 18;(72)*

Figure 2-3 Schematic of the IP-ABE Assay to Purify and Detect Palmitoylation

(1)Washed platelets were lysed by RIPA buffer, and proteins were purified using a specific antibody, and immobilized on protein A-sepharose beads . The purified target protein was then treated with NEM to block free thiol (-SH) groups along unmodified cysteines (C). Then HA pH7.4 (2) was used to specificly cleave thioester bonds at acylated cysteines and generate newly free reactive thiol group (-SH). Next step, the target protein was (3) treated with biotin-BMCC, a sulfhydryl-reactive biotinylation reagent resulting in specific biotinylation of the newly free cysteine. Finally, (4) the biotinylated target protein was eluted in 2% SDS lysis buffer. The acylation of target protein was specificly biotinylated and detected with SDS-PAGE gel, and western blotting with streptavidin-HRP to detect for acylation of the purified t-SNARE proteins.

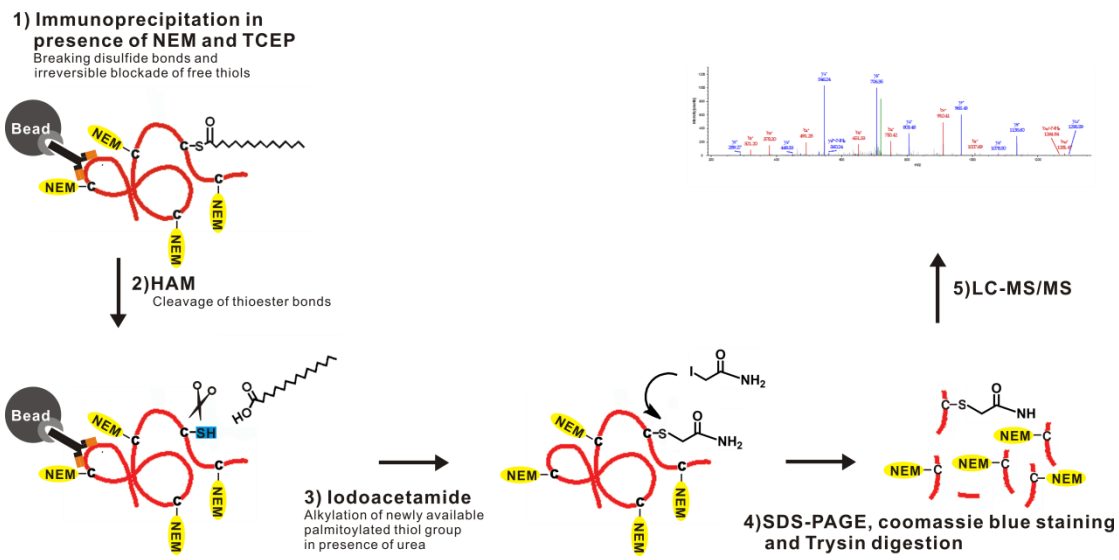


Figure 2-4 Schematic of Mass Spectrometry Analysis of Acylation Sites

Washed platelets were lysed by RIPA buffer, and proteins were purified using a specific antibody, and immobilized on PAS. The purified target protein was then treated with NEM to block free thiol (-SH) groups along unmodified cysteines (C). Then HA pH7.4 (2) was used to specificly cleave thioester bonds at acylated cysteines and generate newly free reactive thiol group (-SH). Next step, the target protein was (3) treated with iodoacetamide, an alkylating agent resulting in specific alkylation of the newly free cysteine. Finally, (4) the alkylated target protein was resuspended in 2% SDS lysis buffer, purified by SDS-PAGE gel and stained by Coomassie blue. Target proteins were recovered and in-gel digested by trypsin overnight at 37 ℃. (5) The digested samples were sent to Mass Spectrometry for analysis.

Chapter Three: SNAP-23 Phosphorylation in Platelet Secretion

Introduction

In platelets, SNARE proteins mediates membrane fusion through several steps: targeting, docking, priming and membrane fusion. Regulatory proteins, such as Munc13-4 and Munc18-2, play an essential role in enhancing membrane fusion (66,69,70,73). In addition, PTM, such as phosphorylation, regulates SNARE-mediated membrane fusion. Phosphorylation of SNAREs has been reported in platelets and has been proposed to be regulated by an array of kinase: *e.g.*, PKC, PKA, IKK and SNAK (80,83,91,174,175). As one Ser/Thr kinase, PKCα regulates many aspects of platelet function, such as activation and thrombus formation (176). PKC can phosphorylate SNAREs and regulatory proteins, such as SNAP-23, SNAP-25, and Munc18-1, thus promoting exocytosis (82,177). Phosphorylation of SNAP-23 at Ser95 and Ser120 has been reported in stimulated mast cells and platelets (83). IKK is the relevant kinase in mast cells and appears to promote SNARE complex (syntaxin-4/SNAP-23/VAMP-8) formation in nongenomic way (99). In platelets, aggregation and granule secretion are inhibited when platelets are treated with IKK-specific inhibitors (80). Mice given IKK inhibitors had significantly prolonged bleeding times showing that IKK activity is important for hemostasis *in vivo* (80). Platelet-specific IKKβ knockout mice also had a defect in platelets secretions and prolonged bleeding. Phosphorylation of SNAP-23 and IκB were decreased in $IKK\beta^{-/-}$ platelets. Combining IKK inhibitor studies and data from the

IKKβ-specific knockout model, it is clear that IKK is important in the regulation of platelet secretion and hemostasis. These defects correlate with the loss of SNAP-23 phosphorylation. However, since all those work were done in animal models or platelets, there is no direct evidence showing that IKK-mediated phosphorylation of SNAP-23 is important for membrane fusion. The first part of my dissertation directly addresses the role of IKK-mediated SNAP-23 phosphorylation in membrane fusion using a liposome fusion assay, purified SNAREs and kinases, and phosphomimetic mutants. I will additionally seek to determine if IKK directly phosphorylates SNAP-23 or promotes a PKC dependent phophorylation.

Results

IKK, but not PKC, directly Phosphorylates SNAP-23: In platelets, SNAP-23 phosphorylation was detected upon activation with several agonists (84). The phosphorylation sites were identified in mast cells and platelets and Ser 95 and Ser 120 appeared to both be modified (83). IKKβ$^{-/-}$ knockout mast cells, SNAP-23 phosphorylation required IKK (99) and similar results were obtained in platelets (80). PKC is the kinase involved in regulating platelet secretion, cytoskeleton reorgnization and spreading, thrombus formation, and platelet activation (178-180). SNAP-23 can be phosphorylated by PKC *in vitro* (84); however, there was no direct evidence to show whether IKK or PKC phosphorylates SNAP-23 *in vivo*. To answer this question, kinase assays were performed. Equal amounts of IKK and PKC were incubated with t-SNARE complex, syntaxin-2/SNAP-23 for 1 hr, and phosphorylation of SNAP-23 was monitored by Western blotting. As shown in Figure 3-1, the degree of SNAP-23

phosphorylation at Ser95 by IKK was 50 fold higher than that by PKC, indicating that SNAP-23 (specifically Ser95) is the substrate of IKK rather than of PKC. In the negative control groups, SNAP-23 was not phosphorylated when either kinase was omitted.

Phosphorylation of SNAP-23 Plays a Role in Membrane Fusion: In Figure 3-1, IKK appears to directly phosphorylate SNAP-23 at Ser95, while PKC does not. Karim *et. al.*, showed that SNAP-23 phosphorylation was diminished and platelet granule secretion was inhibited when the IKK-specific inhibitors, BMS-345541 and TPCA-1 were used. In IKK$\beta^{-/-}$ platelets, SNAP-23 was not phosphorylated and the platelets had a secretion defect (80). These *in vivo* and *ex vivo* results suggest that IKK, through its effects on SNAP-23, is important for hemostasis and thrombosis. In mast cells, SNAP-23 phosphorylation can be regulated by IKK and is important for degranulation (99). However, it is unclear what effect phosphorylation has on SNAP-23 activity, specifically in its role in membrane fusion. To address this question and to determine whether SNAP-23 phosphorylation promotes SNARE complex formation, t-SNARE complex (syntaxin-2/SNAP-23, syntaxin-2 is a surrogate for syntaxin-11) we used an in vitro proteoliposome fusion assay system. Proteoliposomes, containing the t-SNAREs, were pre-incucbated with IKK and then mixed with v-SNARE (VAMP-8) proteoliposomes and fusion was monitored by the time-dependent increase in dequenching of the NBD/rhodamine lipid pair Aliqots of t-SNARE proteoliposomes probed for SNAP-23 phosphorylation by Western blotting with a phosphopeptide-specific antibody. SNAP-23 was clearly phosphorylated by

IKK, and the phosphorylation was blocked by addition of either BMS-345541 or TPCA-1. SNAP-23 phosphorylation was not detected when IKK or the t-SNARE were omitted (Figure 3-3). Membrane fusion did not occur when the t-SNAREs were omitted showing that the increase in fluorescence truly reflected a SNARE-dependent process. Inclusion of IKK enhanced the initial rates of proteoliposome fusion and this enhancement was blocked by addition of either BMS-345541 or TPCA-1 (Figure 3-2). These data are consistent with the effects in intact platelets study (80) and suggest that IKK-mediated phosphorylation SNAP-23 directly affects its ability to promote SNARE-dependent membrane fusion. The effect of the IKK inhibitors verify the need tfor IKK activity in this process.

SNAP-23 Phosphorylation on Ser95 Promotes SNARE Complex Formation and Enhances Membrane Fusion: In thrombin-activated platelets and mast cells, more than one phosphorylation sites were identified in SNAP-23, and it is demonstrated that SNAP-23 is phosphorylated on Ser120 and Ser95 (83). Also, it is shown that IKK phosphorylates Ser120 and Ser95 in mast cells and platelets. Due to the instability of our phosphor-peptide specific antibody that recognizes modification of Ser120, we could not reliably detect SNAP-23 phosphorylation on Ser120. In addition, it is possible that other phosphorylation sites are unidentified in activated platelets, and thus affect membrane fusion. To further confirm the role of SNAP-23 phosphorylation on Ser95 in membrane fusion, site-directed mutagenesis was used to replace Ser95 with negative charge amino acids (Asp and Glu residues) were used to replace Ser95, mimicking a phosphorylation event. Mutation to Ala was used as a negative control.

Mutant SNAP-23/syntaxin-2 were purified and reconstituted into proteoliposomes, and mixed with VAMP-8 containing proteoliposomes to measure fusion. In Figure 3-4, proteoliposomes containing mutant SNAP-23 with mutations of Se95 to Asp95 or Glu95 showed significantly enhanced initial fusion rates as compared to wild-type (WT) SNAP-23-containing proteoliposomes. The Ala95 mutant had a fusion rate similar to WT SNAP-23. In summary, these data suggest a positive role of phosphorylation of SNAP-23 on Ser95, in enhancing membrane fusion.

Discussion

In summary, our data supported the pathway in which IKK phosphorylates SNAP-23 on Ser95 in platelets, and thus enhances membrane fusion in platelets. From our previous work, PKC inhibitors affect SNAP-23 phosphorylation in platelets (80); however, Ser95 phosphorylation of SNAP-23 was not identified in PKCα-activated platelets (179). Our previous studies suggested that a PKC isoform was upstream of IKK, which is consistent with other studies of T- and B-cells (181). SNARE complex (syntaxin-11/SNAP-23/VAMP-8) formation and SNAP-23 phosphorylation were detected in activated platelets by co-immunoprecipitation and Western blotting; however, SNARE complex formation was not detected in platelets pretreated with IKKβ inhibitors or in IKKβ$^{-/-}$ platelets, suggesting that SNAP-23 phosphorylation by IKK promotes or stabilized SNARE complexes and thus promotes membrane fusion upon platelet activation (80).

From these data, both *in vitro* and *in vivo* manipulation of IKKβ activity affects platelet secretion and SNAP-23 phosphorylation, suggesting that IKK activity is

needed or platelet function. At the same time, SNARE complex formation was affected, indicating that phosphorylation of SNAP-23 may be involved in SNARE complex formation and membrane fusion in platelets. The data in this chapter confirm this point since I showed an enhanced fusion rates in IKK treated, SNARE-containing proteoliposomes. However, it is unclear whether phosphorylation of SNAP-23 precedes SNARE complex formation or follows it. While we can show that IKK can phosphorylate monomeric SNAP-23 *in vitro*, it is not clear what the preferred substrate is *in vivo*. Further studies are required to determine how SNAP-23 is phosphorylated -- SNAP-23 monomer, heterodimer syntaxin/SNAP-23, or SNARE complex (syntaxin/SNAP-23/VAMP-8).

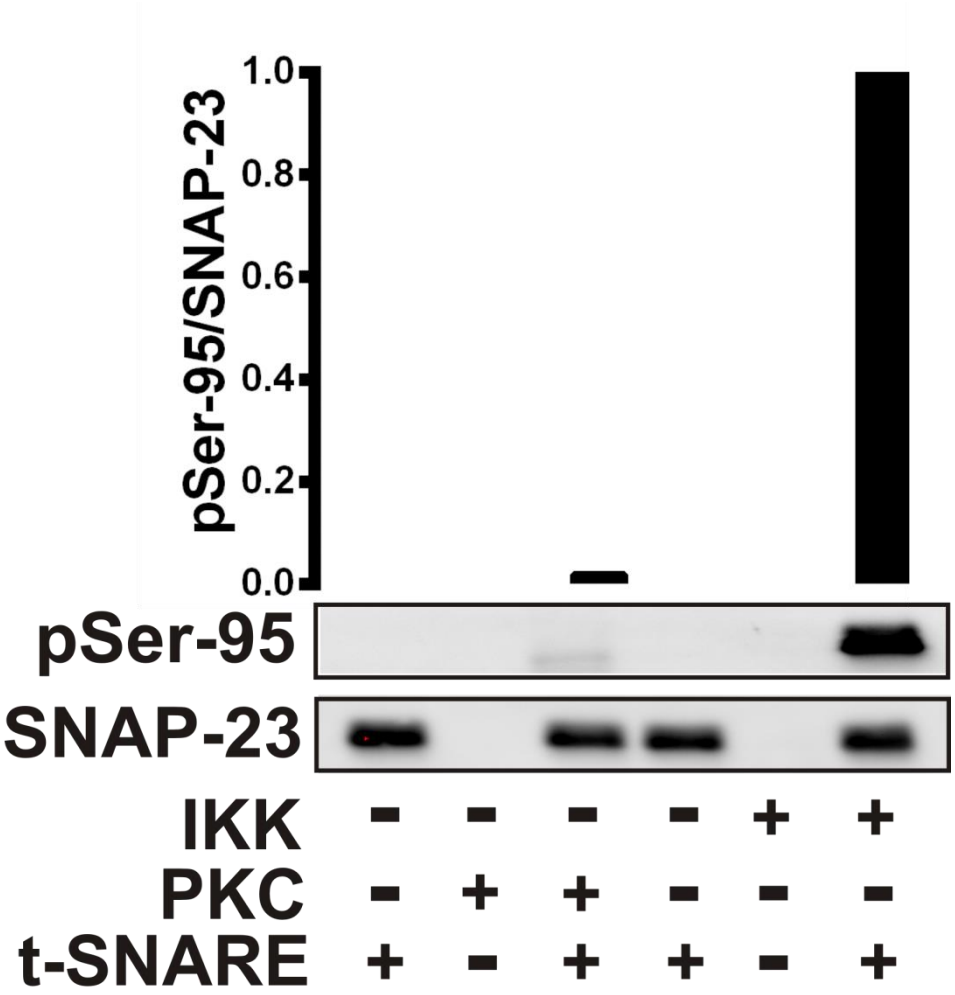


Figure 3-1 PKC and IKK Kinase Affect SNAP-23 Phosphorylation.

t-SNARE proteoliposomes were incubated with 0.5 mM ATP, 10 mM $MgCl_2$, and IKKβ(0.1U/reaction), or PKC (0.1U/reaction) at 30℃ for 1 hr. In the PKC group, 0.1mg/mL DAG (Avanti Polar Lipids), phosphatidylserine (15% PS) and 1mM $CaCl_2$ were present in 20 mM MOPS buffer pH7.4. Na_3VO_4 (0.5mM) and 2.5 mM DTT were also added into the kinase reactions. t-SNARE mixture was subjected to Western blotting using anti-phosph-Ser95 (pSer-95) and SNAP-23 antibodies. Blots were quantified and presented as a ratio of Phospho-SNAP-23/Total SNAP-23. Blots shown are representative of two independent experiments.

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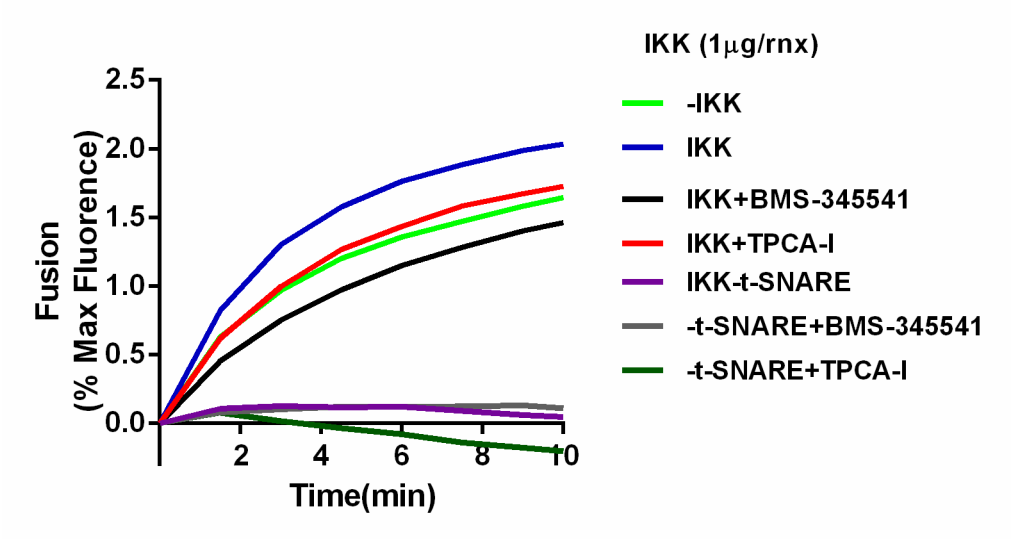


Figure 3-2 IKK Enhances Membrane Fusion and Its Inhibition Blocks the Effect *In Vitro*

SNARE-bearing vesicles, t- and v-SNARE were reconstituted as described in Chapter 2 "Methods" section. t-SNARE vesicles were incubated with 0.5 mM ATP and 10 mM $MgCl_2$ and 1μg/ reaction IKKβ with or without IKKβ inhibitors, BMS-345541 (5 μM) or TPCA-1 (0.5μM) at RT and then were mixed with v-SNARE proteoliposomes. The fusion rates were monitored at 37℃ as an increase in NBD fluorescence. After 1 hr, n-dodecyl-β-D-octylglucoside was added to obtain the maximum NBD fluorescence and the fusion rates were calculated as the percent of that maximum. The data are representative of at least two independent experiments.

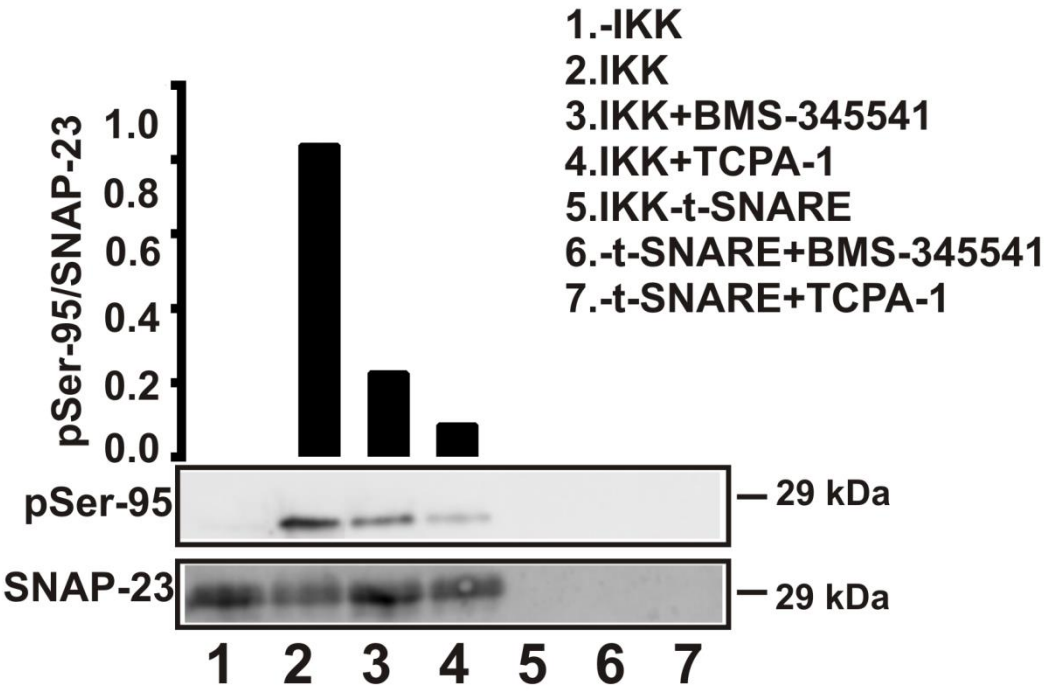


Figure 3-3 IKK Directly Phosphorylates SNAP-23 and Phosphorylation is Blocked by IKK Inhibitors, *In Vitro*

Proteoliposomes bearing t-SNAREs were incubated with 1μg/ reaction IKKβ, and then treated with or without IKKβ inhibitors, BMS-345541 (5 μM) or TPCA-1 (0.5μM) or without IKKβ inhibitors for 60 min (as described in Figure 3-2 legend), a aliquot of the t-SNARE mixture was subjected to Western blotting using anti-phospho-Ser95 (pSer-95) and SNAP-23 antibodies. Blots were quantified and presented as a ratio of pSer-95/Total SNAP-23. The data are representative of at least two independent experiments.

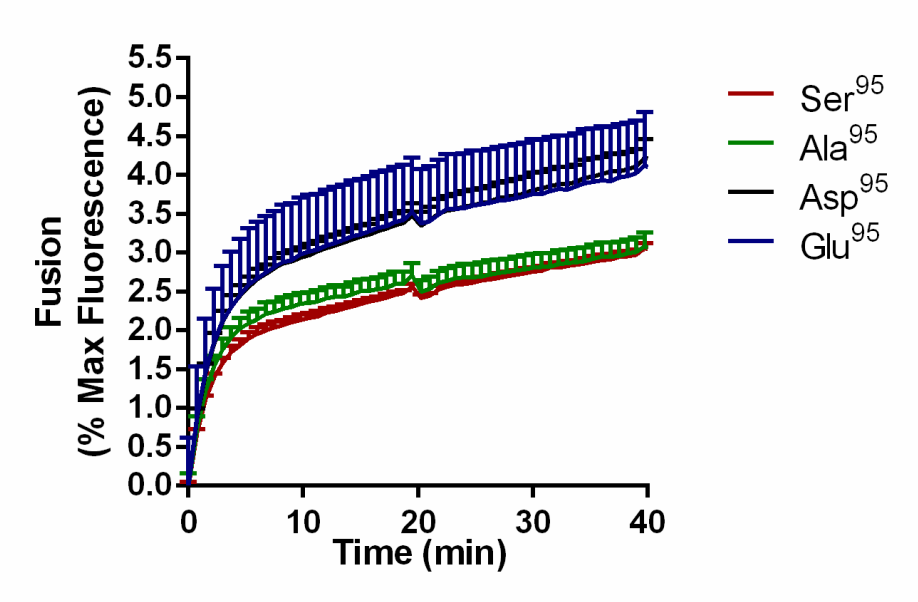


Figure 3-4 Phosphomimetic Residues at Ser95 Affect the Rates of SNARE-Mediated Membrane Fusion

Ser95 in WT SNAP-23 was substituted with Asp, Glu, or Ala and mutant SNAP-23 were purified and reconsistituted into proteoliposomes. Fusion assays were carried out as described in Chapter 2. Muntant SNAP-23/syntaxin-2-bearing proteoliposomes, and VAMP-8-bearing vesicles was reconstituted and preincuated at 37 ℃. After 20 min preincubation at 37 ℃, t-SNARE and v-SNARE were mixed and fusion was monitored at 37 ℃. Ca^{2+} (1 mM final concentration) was added to reactions at t=20 min as indicated. After 60 min, detergent was added to reactions to obtain the maximum NBD fluorescence and fusion was plotted as the percent of maximum NBD fluorescence over time.

Chapter Four: The Role of Acylation in Platelet Secretion

Introduction

Platelet exocytosis is clearly critical for hemostasis. Platelets detect blood vessel injuries and act as tiny building blocks to form a hemostatic plug. Molecules released from platelet granules control the vascular microenvironment underlining why it is critical to understand the mechanism and the regulation of the platelet exocytosis. As with other secretory processes, platelet secretion is driven by protein-protein interactions whereby the SNAREs, v-SNAREs and t-SNAREs, assemble into a four-α-helical bundle that mediates membrane fusion between targeting membrane and vesicles. There are several SNAREs identified in platelets: v-SNAREs, including VAMP-2, -3, -4, -5, -7, -8; and t-SNAREs including, syntaxin-2, -4, -6, -7, -8, -11, -16, -17, -18, SNAP-23, -25, -29 (36,48). Previous work showed that VAMP-8, syntaxin-11, and SNAP-23 serve as primary SNAREs in platelets because their deletion alone leads to a secretion defect (39,50-52,54). Our group and others have also identified a number of SNARE regulators: Munc18-b, Munc13-4, Tomosyn-1 and SLP4s (69,73,78). These regulators are required for granule secretion. However, aside from IKK-mediated phosphorylation of SNAP-23, very little is known about whether other PTMs affect the platelet secretory machinery and thus exert control on exocytosis. In this chapter, I probe the role that acylation plays in controlling two primary t-SNAREs, SNAP-23 and syntaxin-11.

S-acylation is the covalent attachment of an unsaturated fatty acid, palmitate (C_{16})

or other long-chain fatty acids to cysteine residues of proteins *via* a thioester bond. Acylation plays an important role as a membrane anchor and contributes to protein localization, protein stability, and protein-protein interactions (106-109). Acylation can also promote a protein's association with lipid rafts. Acylated proteins partition into membrane rafts where they are known to mediate cell signaling events (182,183). It is also possible that rafts are a site for membrane fusion and granule release (90,120,184,185). S-acylation is enzymatically regulated, by PATs and palmitoyl thioesterases. Though there are numerous PAT's, there are only four thioesterases identified in mammalian cells: acyl protein thioesterase 1 (APT1), acyl protein thioesterase 2 (APT2), palmitoyl protein thioesterase 1 (PPT1), and palmitoyl protein thioesterase 2 (PPT2) (102,127). PPT1 and PPT2 were found in lysosomes and play a role in degradation of palmitoylated proteins (128).

Regulated acylation of SNAREs might affect their role in membrane fusion. In this chapter, one PAT inhibitor and one palmitoyl thioesterase inhibitor were used to study the effects of SNARE acylation in platelets. Cerulenin (2,3 epoxy-4-oxo-7,10 dodecadienamide**)** is a natural product inhibitor of fatty acid synthesis and protein palmitoylation (157). It irreversibly alkylates cysteines in PATs, preventing acyl-enzyme adduct formation (158). It was also shown to inhibit platelet aggregation and thrombus formation *in vivo* (102). Palmostatin B is an inhibitor of β-lactone acyl protein thioesterase 1. In the presence of palmostatin B, the proper localization of H-Ras and N-Ras ares disrupted, and therefore the signaling pathway is down-regulated in nucleated cells (165).

As primary t-SNAREs, syntaxin-11 and SNAP-23 lack a stretch of hydrophobic residues long enough to serve as a membrane anchor (40). There is no CAAX motif found in either sequence, so farnesylation (C_{15}) or geranylgeranylation (C_{20}) are not likely to happen. Both t-SNAREs contain cysteine-rich regions: syntaxin-11 has 8 cysteines, with 6 at its C-terminus (its cysteine-rich regions); and SNAP-23 contains 6 cysteines, with 5 in its central domain (its cysteine-rich regions). In proteomic analysis, Louisa *et al.* identified 215 high confidence palmitoylated proteins in platelets. Syntaxin-11 is one of the novel candidates. SNAP-23 is known to be palmitoylated in its highly conserved cysteine-rich domain between its N- and C-terminal SNARE motifs (119). Acylation of SNAP-23 was shown in platelets (102). Consistently, Sim *et al.* showed that PAT inhibitor pre-treatment blocked platelet activation in response to several hemostatic agonists (102). Pre-treatment of permeabilized platelets with a recombinant thioesterase released SNAP-23 from membranes. However, despite these insights, the nature and potential importance of t-SNARE acylation, specifically of syntaxin-11 and SNAP-23, is largely undefined in platelets.

In this Chapter, we probed the role of SNARE acylation in platelets using chemical and functional assays. We showed that acyl groups were attached *via* thioester linkages that were sensitive to HA. Radiolabeled palmitate was readily incorporated into the two t-SNAREs in metabolic labeling experiments suggesting a rapid turnover of acyl groups, since there is minimal protein synthesis in resting platelets. The extent of acylation did not appear to change upon stimulation or upon

passivation. The acylation sites in these two t-SNAREs were identified by mass spectrometry and shown to be in the predicted cysteine-rich regions. The t-SNAREs did associate with lipid rafts and were enriched in those fractions upon activation. A PAT inhibitor blocked stimulated platelet secretion in a time- and dose-dependent manner which was reversed by pretreatment with a thioesterase inhibitor. Taken together, the data in this chapter show that the two platelet t-SNAREs, Syntaxin-11 and SNAP-23, are dynamically S-acylated and that this turnover of acylation is important for platelet exocytosis.

Results

Syntaxin-11 and SNAP-23 are Associated with Membranes and Present in Lipid Rafts: Syntaxin-11 and SNAP-23 both lack TMD so we initially sought to determine the extent to which they associated with platelet membranes. Membrane fractionation was performed and the t-SNAREs in each fraction were assessed by western blotting. As shown in Figure 4-1, syntaxin-11 and SNAP-23 were barely detectible in the cytosol fractions (S1), but were predominately found in the platelet membrane fractions (both Triton X-100 solution (S_{TX}) and insoluble (I_{TX}) fractions). This shows that the t-SNAREs are mainly membrane associated despite the fact that neither possesses a standard TMD. Many of the proteins probed for were solubilized by Triton X-100, but there was a fraction of t-SNAREs present in the insoluble (I_{TX}) fractions. The insoluble (I_{TX}) fractions were prepared by successive centrifugations (at 100,000 × g) and they contained cytoskeletal pellet and lipid rafts (186). Consistent with previous studies in mast cells (83), we found SNAP-23 associated with this

fraction. In mast cells, ternary SNARE complexes (syntaxin-4/SNAP-23/VAMP-2) were enriched in lipid rafts during mast cell exocytosis suggesting that rafts may be the route of exocytosis (90).

To determine whether t-SNARE localization changes upon platelet activation, lipid rafts were prepared from thrombin activated platelets. Depicted in the diagram (Figure 4-2), fractions 9-11 are lipid raft rich fractions. Our data show that syntaxin-11, SNAP-23 and VAMP-8 were present in the lipid raft fractions from resting platelets (R group) and more SNAREs were present in the rafts prepared from activated platelets(S group). Interestingly, there was a significant enrichment of VAMP-8 (1.5-fold increase) in the lipid rafts from activated platelets. This together with the enrichment of syntaxin-11 and SNAP-23 into the rafts of activated platelets is consistent with what is seem in mast cells and suggests that, like in mast cells, the lipid rafts may be the site of membrane fusion in platelets.

Cholesterol Depletion Affects t-SNARE Localization and Platelet Function: Lipid rafts are predominantly composed of cholesterol and sphingolipids which are thought to exist as aggregates in the lipid bilayer. Cholesterol acts as a spacer between hydrocarbon chains of the sphingolipids and aids in the assembly of the raft aggregates (168). Depletion of cholesterol can cause proteins to dissociate from lipid rafts (187). The data in Figure 4-2 shows that the t-SNAREs are present in lipid rafts. To determine whether cholesterol affects this distribution and thus affects platelet functions, cholesterol was depleted using methyl-β-cyclodextrin (MβCD). With the strategy, total platelet cholesterol was reduced more than 50% (Figure 4-5).

Consistently, on the sucrose gradients less SNARE protein (syntaxin-11, SNAP-23 and VAMP-8) was present in the lipid raft fractions from the MβCD treated -platelet compared to untreated controls (Figure 4-3). Accordingly, platelet aggregation and ATP release were inhibited in cholesterol-depleted platelets when either A23187 or thrombin was used as agonists (Figure 4-4). Since cholesterol deficiency had a negative effect on platelets, we asked the converse, does increased cholesterol positively affect platelets? For this, platelets from apolipoprotein E (ApoE) knockout mice were examined. Total platelet cholesterol was elevated by 35% in $ApoE^{-/-}$ platelets compared with that in WT platelets (Figure 4-6). Washed platelets from the $ApoE^{-/-}$ showed a significantly greater response to stimulation with thrombin. Both ATP release and platelet aggregation were hyper-responsive when compared to WT mouse platelets. Overall, cholesterol, as one major components of lipid rafts, appears to be essential for the raft association of the SNARE and is also important for platelet function.

Acylation and Metabolic Incorporation of [^{3}H]-Palmitic Acid into Platelet Syntaxin-11 and SNAP-23: In Figure 4-1, syntaxin-11 and SNAP-23 act as integral membrane proteins though they lack TMDs. PTMs, such as acylation or prenylation, could serve as a lipid anchor. Neither t-SNARE contains a CAAX box but both contain a cysteine-rich region that could undergo S-acylation. To address this point, we used the IP-ABE assay in which the acyl groups on immuno-precipitated proteins are first cleaved with hydroxylamine and then replaced with a biotinylated maleimide derivative. Figure 4-7 shows that syntaxin-11 and SNAP-23 from platelets can be

detected in this assay. HA specifically cleaves thioester linkage at neutral pH (pH7.5), and once removed, the biotin-BMCC covalently modifies the exposed cysteine forming a biotinylated protein. In the absence of HA, no biotinylated syntaxin-11 and SNAP-23 was detected. These data are consistent with previous studies of these two t-SNAREs in other systems (46,112).

Since platelets are anucleate, they have a very limited capacity for proteins synthesis. Most of proteins present in platelets are synthesized at the megakaryocyte stage. However, it seems possible that the thioester-linked acylation could turn over in resting platelets and those might be dynamic. To address this question, washed human platelets were incubated with radioactive palmitate for 1 hr or 2 hr. [^{3}H]-Palmitic acid efficiently incorporated into both syntaxin-11 and SNAP-23 (Figure 4-8A) in a time-dependent manner. To demonstrate that [^{3}H]-palmitic acid was incorporated into the proteins *via* a thioester bonds, 2.5% HA at neutral pH was incubated with IP samples (syntaxin-11 and SNAP-23) for 30 min prior to electrophoresis and autofluorography (Figure 4-8B and C). Both syntaxin-11 and SNAP-23 lost their [^{3}H]-palmitic acid. Based on the HA-treated samples, the labeled palmitic acid was attached to the t-SNARES via an hydroxylamine-sensitive thioester bond.

Analysis of Acylation Sites in Syntaxin-11 and SNAP-23 by Mass Spectrometry: Given our data and the reversibility of S-acylation it is likely that acylation turns over in platelets and thus some of the t-SNAREs might be incompletely acylated. Therefore, we asked whether all the cysteine of each t-SNARE can be acylated? In addition to the cysteine-rich regions of t-SNAREs, there are additional cysteines in

syntaxin-11 and SNAP-23 that were also accounted for. To detect each acylation site, we used two alkylating reagents, Iodoacetic acid (IAA) and N-ethylmaleimide (NEM), in combination with HA treatment and tryptic peptide mass spectrometry. Immunopurified proteins were treated with TCEP to reduce any disulfide bonds and the free cysteines were covalently modified with NEM. The proteins were then treated with HA to cleave thioester-linked moieties and the exposed cysteines were modified with IAA. For analysis, the proteins were cleaved with trypsin and subjected to mass spectrometry. For syntaxin-11 (n=2), we found evidence of NEM modifications on Cys102 (GEVIH$_{\mathbf{NEM}}$**C**$^{\mathbf{102}}$K; monoisotopic *m/z* = 455.73005 Da; Figure 4-11) and Cys157 (QRDN$_{\mathbf{NEM}}$**C**$^{\mathbf{157}}$KIR; monoisotopic *m/z* = 579.302623 Da; Figure 4-12). No other NEM modified peptides were identified suggesting that these two Cysteines were either free or involved in a disulfide bond. Upon HA treatment and IAA modification, we detected (n=2) two tryptic peptides that were modified: KAVQYEEKNP$_{\mathbf{IAA}}$**C**$^{\mathbf{275}}$R (monoisotopic *m/z* = 507.92435 Da; Figure 4-10) and TL$_{\mathbf{IAA}}$**C**$^{\mathbf{279}}$$_{\mathbf{IAA}}$**C**$^{\mathbf{280}}F_{\mathbf{IAA}}$**C**$^{\mathbf{282}}$$_{\mathbf{IAA}}$**C**$^{\mathbf{283}}P_{\mathbf{IAA}}$**C**$^{\mathbf{285}}$LK (monoisotopic *m/z* = 759.81061 Da; Figure 4-9). These data are consistent with Cys 275, 279, 280, 282, 283, and 285 being modified by a HA-sensitive thioester-linked, moiety, suggesting that all six Cysteines are S-acylated. For SNAP-23 (n=2), NEM modified only Cys112 (TTWGDGGENSP$_{\mathbf{NEM}}$**C**$^{\mathbf{112}}$NVVSK; monoisotopic *m/z* = 938.41953 Da; Figure 4-14). After HA treatment and IAA modification, we detected (n=2) one modified, tryptic peptide: $_{\mathbf{IAA}}$**C**$^{\mathbf{79}}$$_{\mathbf{IAA}}$**C**$^{\mathbf{80}}GL_{\mathbf{IAA}}$**C**$^{\mathbf{83}}V_{\mathbf{IAA}}$**C**$^{\mathbf{85}}P_{\mathbf{IAA}}$**C**$^{\mathbf{87}}$NR (monoisotopic *m/z* = 728.27869 Da; Figure 4-13). These data are consistent with Cys 112 being either as free

sulfhydryls or in a disulfide bond as part of a homo- or hetero-dimer and Cys 79, 80, 83, 85, and 87 being modified by a HA-sensitive thioester-linked, moiety, consistent with these Cysteines being the site of S-acylation on SNAP-23.

Acylation of t-SNARE during Platelet Activation and Passivation: Acylation and deacylation cycles can be regulated by the opposing actions of PATs and palmitoyl thioesterases, which would account for the turnover in acylation that appears to describe our results. Is this turnover affected by platelet activation state? To answer this point, we examined the incorporation of [^{3}H]-palmitic acid into platelet proteins under activating conditions, with thrombin, and under passivating conditions with PGI_2. In Figure 4-15A and B, [^{3}H]-palmitic acid incorporation into syntaxin-11 and SNAP-23 was unchanged in the presence of PGI_2. It is also unchanged when platelets were activated with thrombin (0.1U/mL). Acylation was inhibited in cerulenin-treated platelets. Cerulenin is an acylation inhibitor in neurons and platelets (102,188). Notably, the levels of syntaxin-11 and SNAP-23 protein remained unchanged suggested that neither new protein was made nor the SNAREs were degraded during the experiment. These data further confirm that the acyl groups are actively turning over on the t-SNAREs' cysteines. To confirm the effects of cerulenin on t-SNARE acylation, the IP-ABE assay was performed. Compared to the vehicle-treated group, acylation of syntaxin-11 and SNAP-23 was decreased in platelets pre-treated with cerulenin (Figure 4-16A and B). Consistently, there is no difference in protein levels between cerulenin- and vehicle-treated platelets**.** Overall, these [^{3}H] palmitate pulse-chase experiments highlight the reversibility of S-acylation and show that it can

be manipulated with the acylation inhibitor, cerulenin.

PAT and APT Inhibitors Affect Platelet Secretion and Activation: In the studies of Sim *et al*., cerulenin was used to analyze the effects of protein acylation in platelets. They showed that cerulenin pretreatment inhibited platelet aggregation (102). We expanded on these original studies by looking at other aspects of platelet function. Cerulenin treatment (56μM, 167μM, 500μM) had little to no effect on thrombin-induced calcium transients as measured with Fura-2 (Figure 4-22). These would suggest that cerulenin has no significant effect on the signaling processes that facilitate thrombin induced increases in intra-platelet [Ca^{2+}], at the concentrations of thrombin used. The effect of cerulenin on platelet aggregation was time-dependent; platelets, pre-treated with cerulenin, were inhibited within 1 hr (Figure 4-18). Cerulenin's effects on aggregation and ATP release were also dose-dependent (Figure 4-17). Among three different concentrations of cerulenin, platelets pre-treated with 500 μM cerulenin displayed the greatest inhibition in ATP release and aggregation. Our data confirmed the results of Sim *et al.* (102).

To examine the effects of cerulenin of α-granule secretion FACS analysis of CD62 (P-Selectin) was used (Figure 4-19). P-selectin exposure, as a metric of from α-granule release, in response to thrombin and A23187 was also inhibited by cerulenin treatment. The effect of cerulenin on A23187-stimulated exocytosis is of note since the calcium ionophore largely bypasses the signaling steps required for agonist-induced platelet activation. The fact that cerulenin inhibits A23187-induced platelet secretion strongly suggests that acylation is directly important for t-SNARE

function in membrane fusion, and not solely required for upstream signaling processes as is implied by Sim *et al.* (102).

The acyl-protein thioesterase (APT) inhibitor, palmostatin B inhibits deacylation. Given that our data with cerulenin-treatment showed that acylation is important for platelet function, we sought to determine if deacylation might contribute to the process. To study the cycle of SNAP-23 and syntaxin-11 acylation, palmostatin B and/or cerulenin were added to washed platelets at different stages of the incubation period. First, acylation was measured from immunoprecipitated t-SNARE proteins using the IP-ABE assay. Unlike what was seen with cerulenin-treatment, palmostatin B enhanced acylation on syntaxin-11 and SNAP-23 compared to vehicle-treated platelets (Figure 4-21). Palmostatin B, unlike cerulenin, appeared to have no effect on platelet secretion in response to thrombin or A23187 Figure 4-20. These data suggested that there was a thioesterase-mediated removal of the t-SNARE acyl groups and that these groups must be replaced to retain platelet function. To address this point, palmostatin B and cerulenin were added sequentially in different orders. While pretreatment with cerulenin prior to palmostatin B inhibited platelets, the converse (palmostatin B treatment first) restored platelet activity. These data would suggest a constant cycle to thioesterase mediated acyl group removal that is counteracted by PAT activity. It is this reacylation that appears to be important for maintaining functionally active platelets.

Acylation is not the Only Factor Affecting Membrane Association: Acylation provides t-SNAREs with lipid anchors, assisting their membrane association; however

there could be other factors that mediate membrane association. To address this point we examined protein distributions after chemical deacylation with HA. In Figure 4-24, HA treatment released very little of the two t-SNAREs from membranes After chemical deacylation HA, syntaxin-11 and SNAP-23 still stayed in the detergent phase of the Triton X-114 partitioning, indicating that their hydrophobicity had not changed compared to control group (no HA treatment; Figure 4-23). In Figure 4-25, syntaxin-11 and SNAP-23 were still present in lipid rafts after cerulenin treatment. Overall, these results indicate that deacylation may not be sufficient to completely release t-SNAREs from membranes. Other factors, such as protein-protein interaction, or some unidentified modifications of these two proteins may affect their membrane association. Consistently, in PC12 cells, acylation is essential for SNAP-25 initial targeting to membrane, but its membrane association can be maintained by protein-protein interactions after deacylations (117).

Discussion

From our data, the S-acylation/deacylation cycle in platelets appears to be dynamic and very important for platelet exocytosis. Thioesterases are actively removing the acyl groups on the two t-SNAREs and PATs appear to be replacing them. Hence we observed a time-dependent incorporation of [^{3}H]-palmitic acid into the two proteins. This incorporation was not altered by platelet activation state since there was no significant increase in [^{3}H]-palmitic acid incorporation into syntaxin-11 and SNAP-23 upon platelet activation by thrombin. There was minimal enhancement of incorporation into total lysates. Previously, [^{3}H]-palmitic acid incorporation into

two unidentified, 38 kDa and 23 kDa proteins was reported in platelets and the incorporation was enhanced upon activation with thrombin within. Incorporation reached a plateau after 15 min and other agonists, *i.e.*, collagen and A23187, had no effect (189). In experiments reported here the incubation times were longer *i.e.*, 1-2 hr, and there was a time-dependence despite being longer than the originally reported incorporation plateau time (15 min). Syntaxin-11 and SNAP-23 are less abundant than the originally detected 38 kDa and 23 kDa proteins; despite the coincident molecular weights, immuno-depletion studies suggested neither t-SNARE was one of these original two acylated proteins.

2-Bromopalmitate (2BP) was used as another acylation inhibitor in β cells, platelets as well as in other cells (159,190). However, in our experience when washed platelets or PRP were treated with 10μM-100μM 2BP, the platelets appear to be solubilized or destroyed and thus it was not used in our studies. Since 2BP contains a long chain fatty acid, perhaps it acts as a detergent but this was not addressed experimentally.

The fusogenic activity of the SNAREs could depend on the number of acyl groups attached to the cysteine-rich regions, this has been seen in PC12 cells with SNAP-23 (119). Even after replacing the TMDs of Syntaxin-1 and VAMP-2, with the cysteine-rich region of syntaxin-19, the two SNAREs were able to efficiently mediate membrane fusion of synaptic vesicle (191). Replacing the Q-SNARE, Nyv1p's TMD with a lipid anchor did not affect its fusogenicity (192). Like syntaxin-19, SNAP-23 and syntaxin-11 both possess cysteine-rich regions instead of TMD and as the extent

of acylation is decreased membrane fusion is affected. From our acylation site analysis (Figure 4-13), it appears that all the cysteine residues in the cysteine-rich regions of both SNAP-23 and Syntaxin-11can be modified by IAA after HA treatment suggesting that all the cysteines can be acylated for at least some population of the SNAREs. We were unable to detect partially modified peptides that contained both NEM and IAA modified peptides. We did however detect cysteines that were most likely involved in disulfide bonds or as free sulfhydryls. These were not in cysteine rich regions.

SNAP-23 and syntaxin-11 are still localized to lipid rafts, even when the platelets are pre-treated with cerulenin (Figure 4-25). At this stage, we cannot evaluate the degree to which the SNAREs are deacylated as a result of the cerulenin treatment so it is difficult to assess how important acylation is to raft association. The raft association would argue that the t-SNAREs still retain some of their acyl groups. Protein-protein interaction may also keep them associated with the membranes and lipid rafts. Acylation is recognized as a modification that promotes association with lipid rafts (90,120). It is interesting to note that in resting platelets there is only partial localization of syntaxin-11 and SNAP-23 to the lipid rafts of platelets. This distribution changes upon platelet activation where even more of the SNAREs are mobilized to lipid rafts. While acylation assists in raft association, we only see a minor change in the extent of SNARE acylation upon activation, suggesting that some other factor(s) might affect raft association. Perhaps raft association is controlled by

both acylation and some protein-protein interaction that is responsive to platelet stimulation. Further experiments will be required to address this point.

Overall, protein acylation appears to be important for SNARE-mediated exocytosis from activated platelets. It may not be the determining factor for their membrane association but it could affect partitioning into rafts and thus SNARE localization. Additionally, since the acyl groups appear to turnover rapidly, the degree and composition of the acyl groups may be highly sensitive to the Acyl-CoAs present in platelets, thus accounting for the increase platelet reactivity seen in patients with dyslipidemia. These are future questions that are derived for the experiments in this chapter.

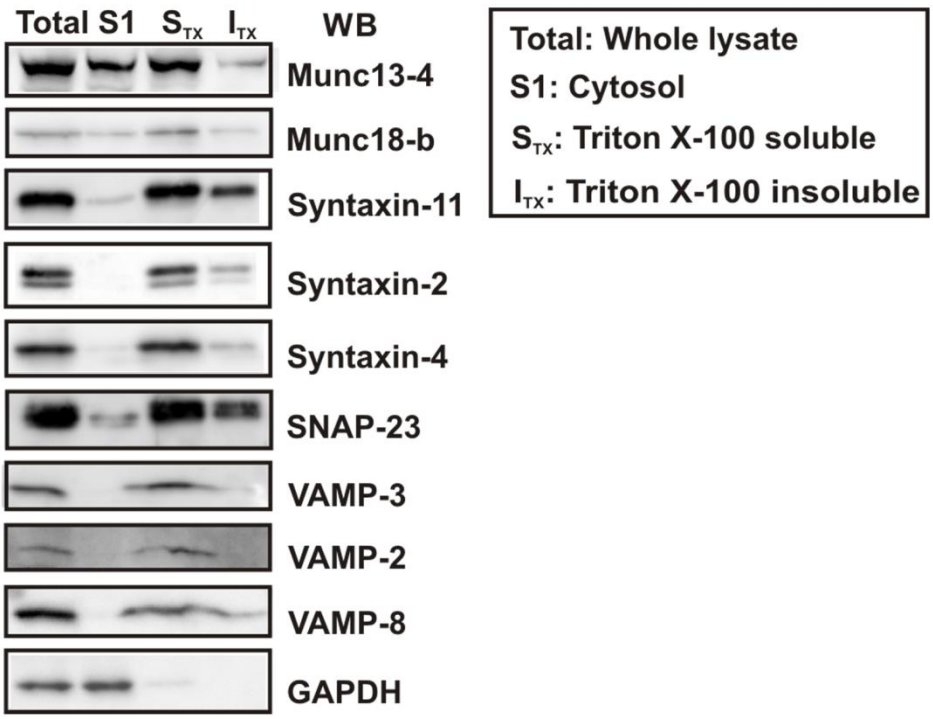


Figure 4-1 Syntaxin-11 and SNAP-23 are Associated with Platelet Membranes

Total lysates were prepared from washed platelets by 5 freeze-thaw cycles and were subjected to ultracentrifugation at 100,000 × g for 1 hr at 4℃ to separate plasma membrane and cytosol fractions. The membrane fractions treated with 1% Triton X-100 for Triton X-100-soluble (S_{TX}) and insoluble fractions (I_{TX}). Equivalent amounts of fractions were analyzed by SDS-PAGE and probed by western blotting with the indicated antibodies. Blots shown are representative of three independent experiments.

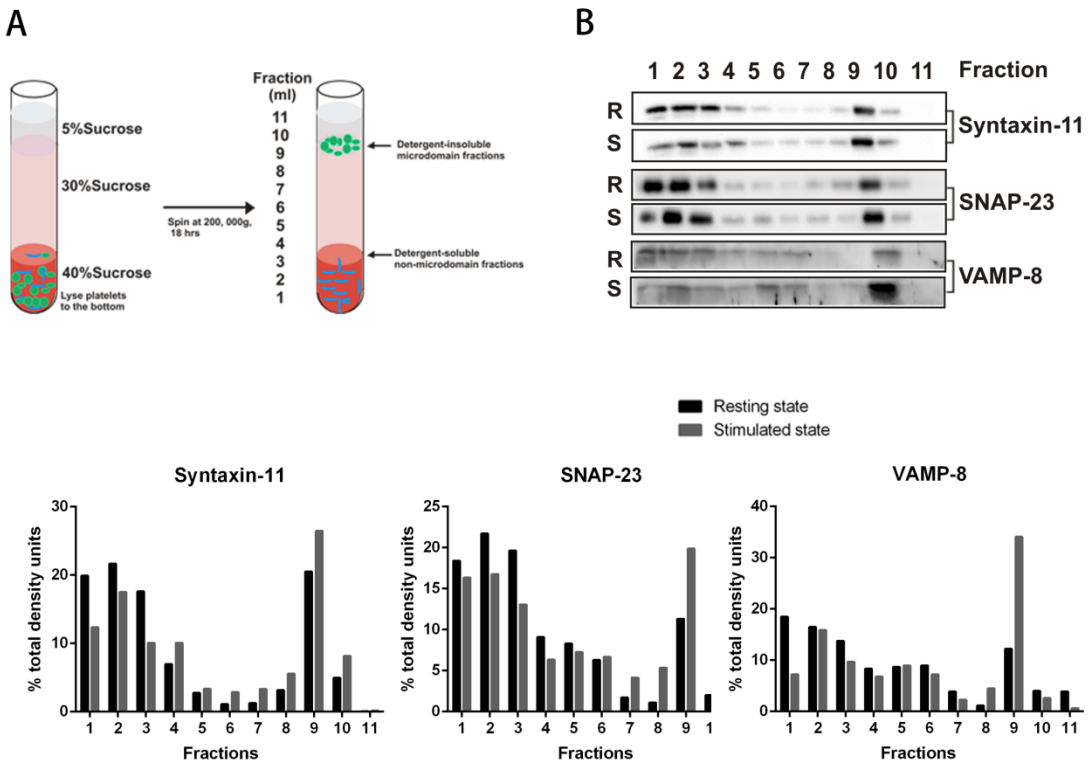


Figure 4-2 Syntaxin-11 and SNAP-23 Are Present in Lipid Rafts Increases Upon Platelet Activation

A). Resting (R) or thrombin-stimulated (S) platelets were lysed in 2 × raft lysis buffer, then mixed 1;1 with 1.5 mL 80% sucrose in MBS (final sucrose concentration is 40%) and layered into ultracentrifuge tubes. 6 mL 30% sucrose were layered on 40% mixture, and finally 2 mL 5% sucrose were layered on the top. The samples were subjected to ultracentrifugation at 200,000 × g for 18 hr at 4 ℃. B). The gradients were fractionated and probed for the indicated proteins by western blotting. Top: Blots shown are representative of three independent experiments. Bottom: Quantifications of the specific proteins in each fraction were shown.

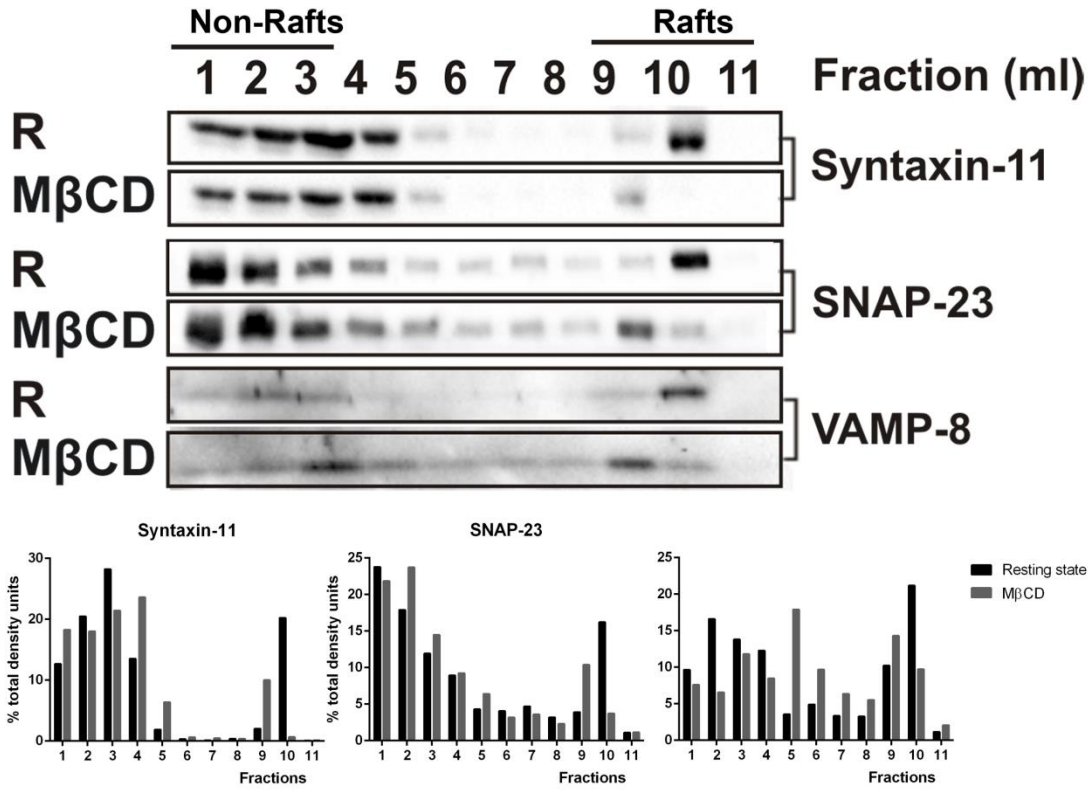


Figure 4-3 Cholesterol Depletion Affects t-SNARE Localization

Washed platelets (1 × 10^9/mL) were treated with 10 mM Methyl-β-cyclodextrin (MβCD) for 15 min at 37°C. Untreated (R) or MβCD-treated platelets were lysed in 2× raft lysis buffer and layered into ultracentrifuge tubes and c The samples were subjected to ultracentrifugation at 200,000 × g for 18 hr. The gradients were fractionated and probed for the indicated proteins by western blotting. Blots shown are representative of three independent experiments. Quantifications of specific proteins in each fraction are shown.

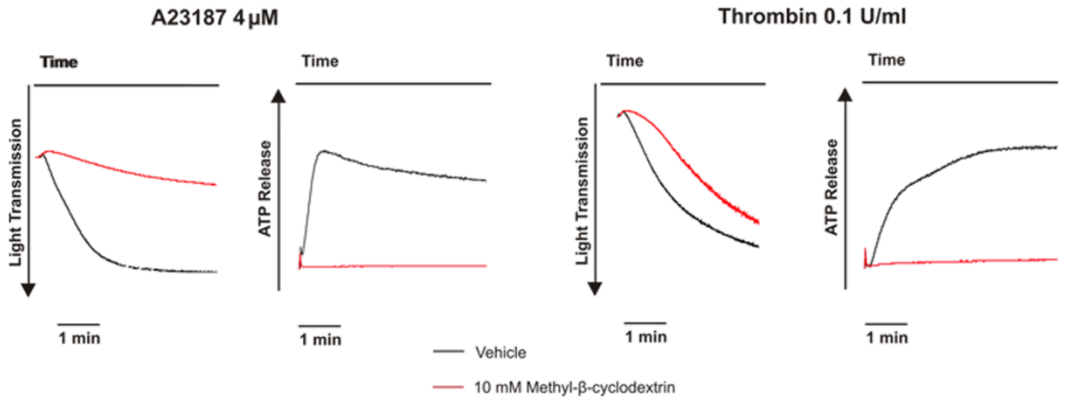


Figure 4-4 Cholesterol Depletion Affects Platelet Secretion and Aggregation

Washed platelets (4 × 10^8/mL) were treated with or without10 mM Methyl-β-cyclodextrin (MβCD) for 15 min at 37°C. Then platelets were stimulated with 4µM A23187 and 0.1U/mL thrombin. ATP release and aggregation were recorded by lumi-aggregometry. Data shown are representative of at least three experiments.

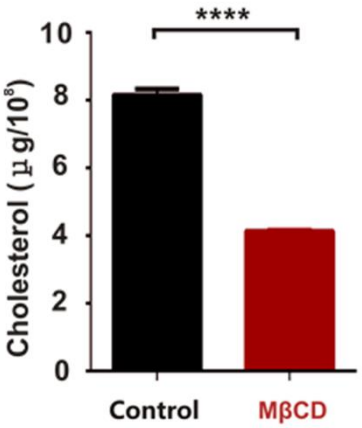


Figure 4-5 Methyl-β-cyclodextrin Treatment Depletes Platelet Cholesterol

The lipids from washed human platelet lipids were extracted with 200 µL chloroform: isopropanol: NP-40 (7:11:0.1) and centrifuged to remove non-lipid fraction. Platelet cholesterol was determined using a cholesterol oxidase assay (Amplex Red Cholesterol Assay Kit, Invitrogen, Life technologies, Grand Island, NY) and converted into µg/10^9 platelets. Data were expressed as mean ± standard deviation. N=9 for each group, ****p<0.0001, using an unpaired student's t-test.

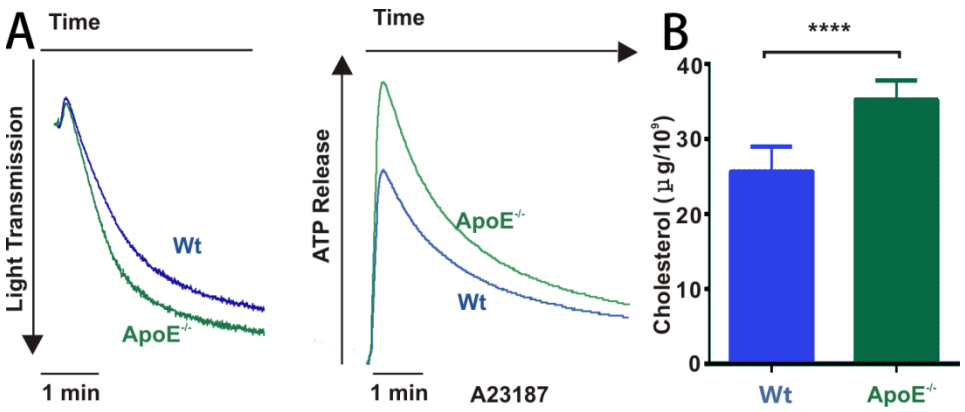


Figure 4-6 Aggregation, ATP Release and Cholesterol Levels are Increased in ApoE$^{-/-}$ Platelets

A). Washed platelets (4×10^8/mL) from WT and ApoE$^{-/-}$ mice were stimulated with 10 µM A23187. ATP release was monitored in a Lumi-Aggregometer. (Tracings are representative of triplicate). B). Platelet lipids were extracted with 200 µL chloroform: isopropanol: NP-40 (7:11:0.1) and centrifuged to remove non-lipid fraction. Platelet cholesterol was determined using a cholesterol oxidase assay (Amplex Red Cholesterol Assay Kit, Invitrogen, Life technologies, Grand Island, NY) and converted into µg/10^9 platelets. Data are expressed as mean ± standard deviation. N=9 for each group, ****p<0.0001, using an unpaired student's t-test.

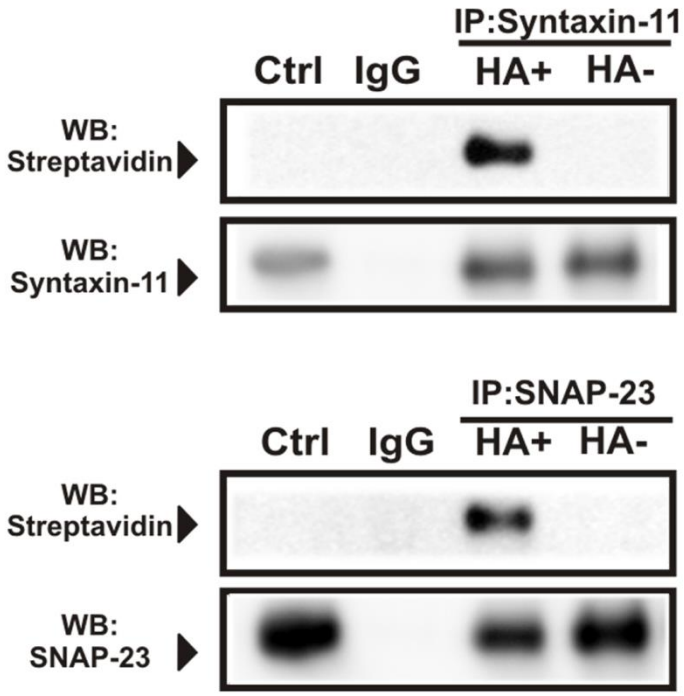


Figure 4-7 Syntaxin-11 and SNAP-23 are Acylated in Platelets.

Washed platelets (1×10^9/mL) were lysed with ice cold 1 × Lysis Buffer containing 50 mM NEM at 4℃ for 60 min. Anti-syntaxin-11 and anti-SNAP-23 Abs were added into lysates and incubated at 4℃. After overnight-incubation, IP samples were treated with 0.1 M HA for 30 min prior to biotinylation with BMCC-biotin. The omission of HA was used as a negative control. The samples were separated by SDS-PAGE and probed for anti-syntaxin-11 and anti-SNAP-23 antibodies by western blotting and for biotinylation with streptavidin-alkaline phosphatase conjugate. Blots shown are representative of three independent experiments.

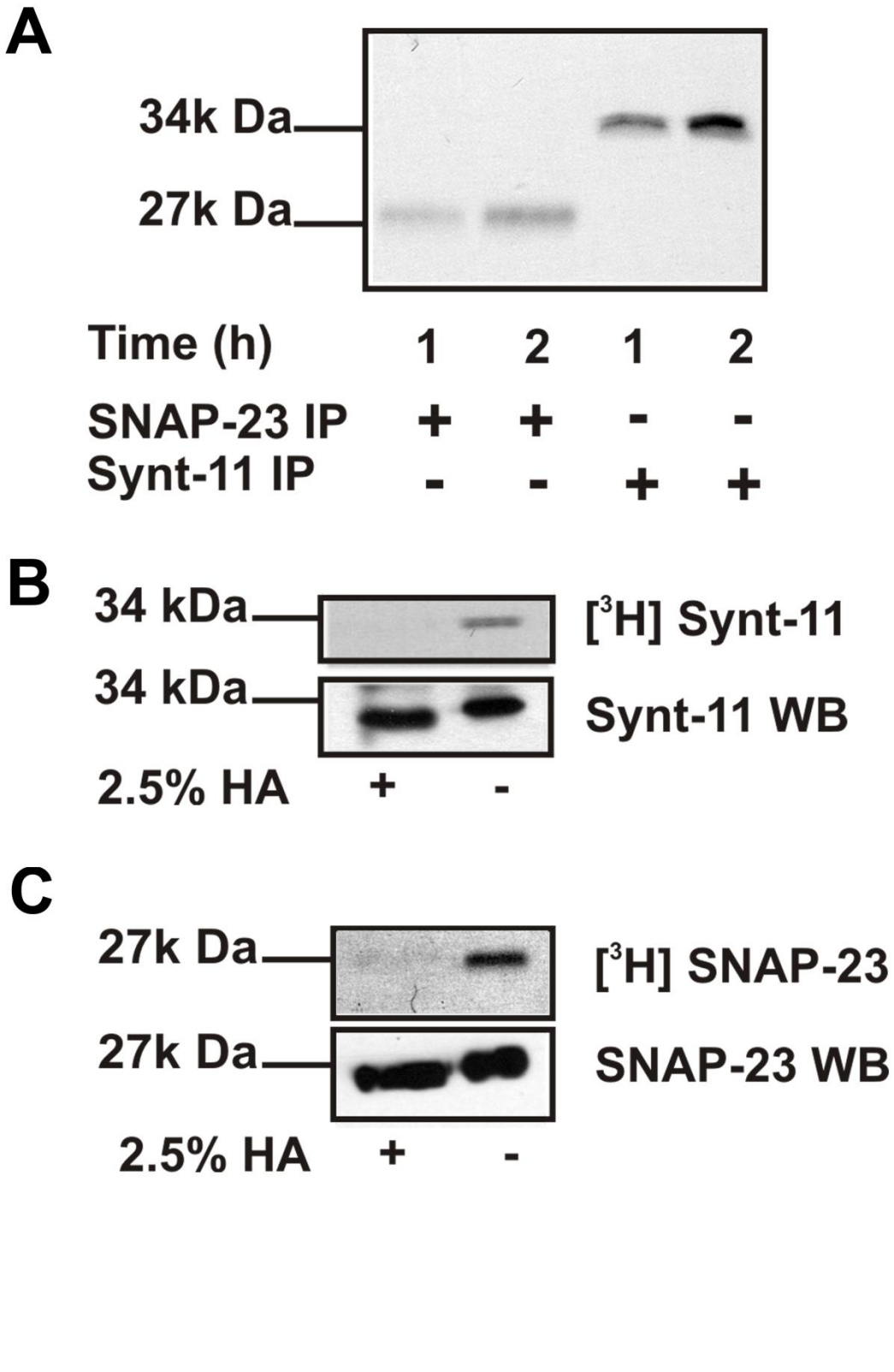

A
34k Da
27k Da
Time (h) 1 2 1 2
SNAP-23 IP + + - -
Synt-11 IP - - + +
B
34 kDa
34 kDa
[3H] Synt-11
Synt-11 WB
2.5% HA + -
C
27k Da
27k Da
[3H] SNAP-23
SNAP-23 WB
2.5% HA + -

Figure 4-8 [^{3}H]-Palmitic Acid Incorporation into Platelet Syntaxin-11 and SNAP-23

A). Human washed platelets were incubated with [^{3}H]-palmitic acid for 2 hr, platelet lysates were subjected to IP with anti-SNAP-23 and anti-syntaxin-11 Abs and the proteins were visualized as above; B). and C). 2.5% HA were incubated with [^{3}H]-palmitate-labeled immunoprecipitated samples. HA treated immunoprecipitated proteins were separated by SDS-PAGE and visualized by autofluorography. The aliquots of HA treated samples were also probed by western blotting with anti-syntaxin-11 and anti-SNAP-23 Abs. Blots or data shown are representative of at least two independent experiments.

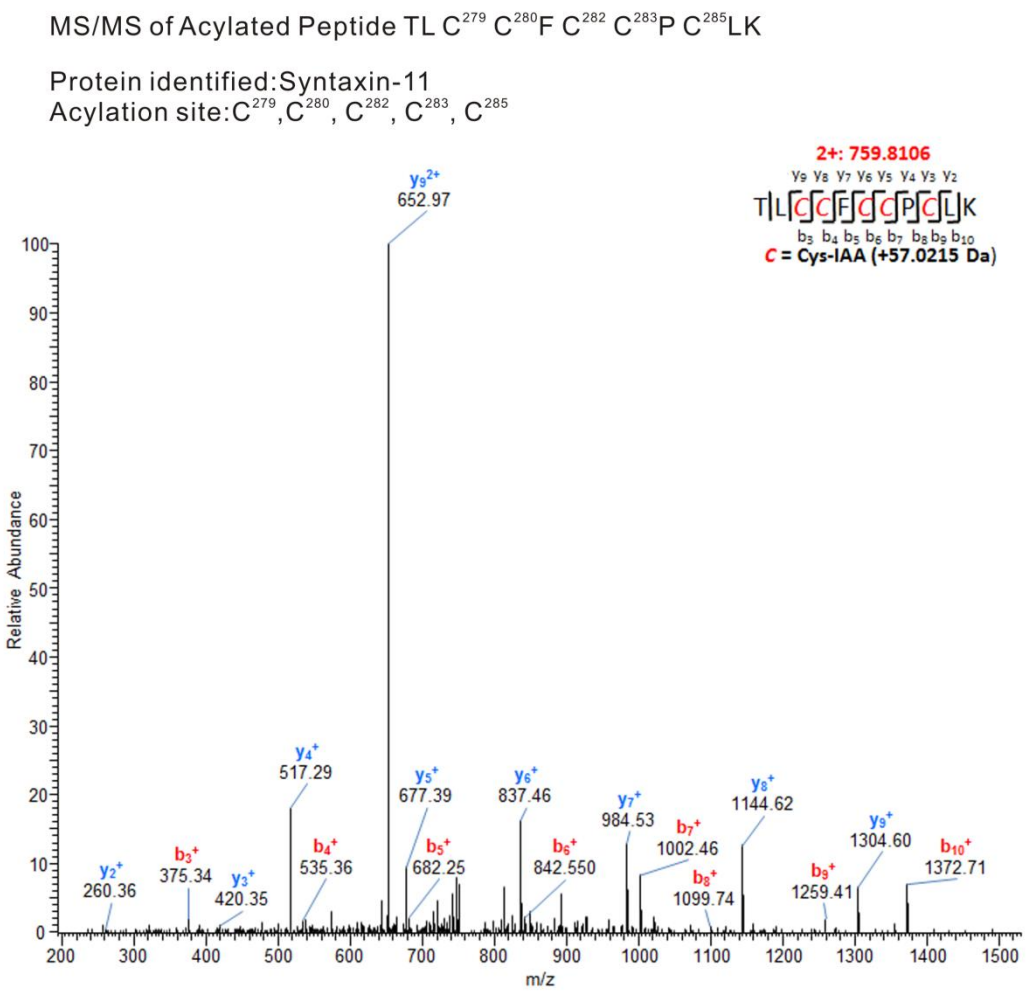


Figure 4-9 MS/MS of the Acylated Peptide from Syntaxin-11: TLC^{279}C^{280}FC^{282}C^{283}PC^{285}LK

Mass spectrums were shown with IAA modified for detecting acylation sites in acylated peptide TLC^{279}C^{280}FC^{282}C^{283}PC^{285}LK of syntaxin-11. Modification sites were identified based on MS/MS fragmentation patterns. For clarity, only *y* and *b* ions are shown. The *y* ions are labeled in blue, the *b* ions in red.

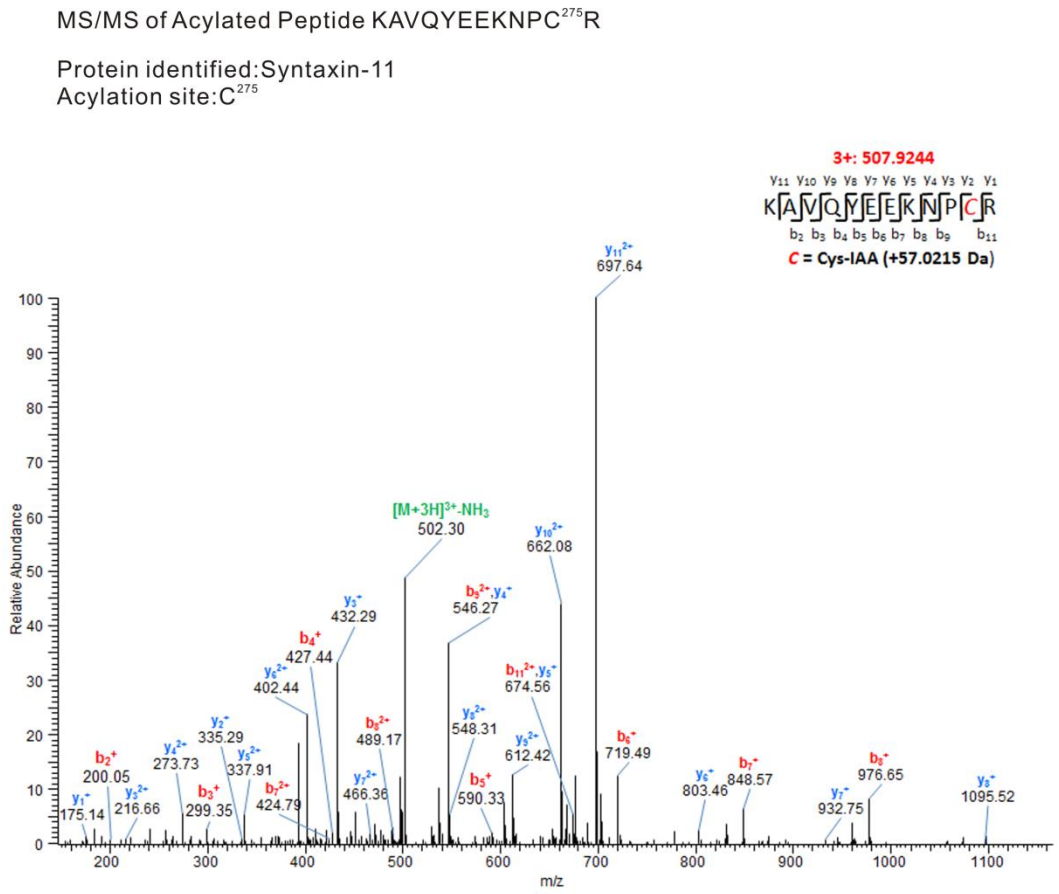


Figure 4-10 MS/MS of the Acylated Peptide from Syntaxin-11: KAVQYEEKNPC^{275}R

Mass spectrums were shown with IAA modified for detecting acylation sites in acylated peptide KAVQYEEKNPC^{275}R of syntaxin-11. Modification sites were identified based on MS/MS fragmentation patterns. For clarity, only *y* and *b* ions are shown. The *y* ions are labeled in blue, the *b* ions in red.

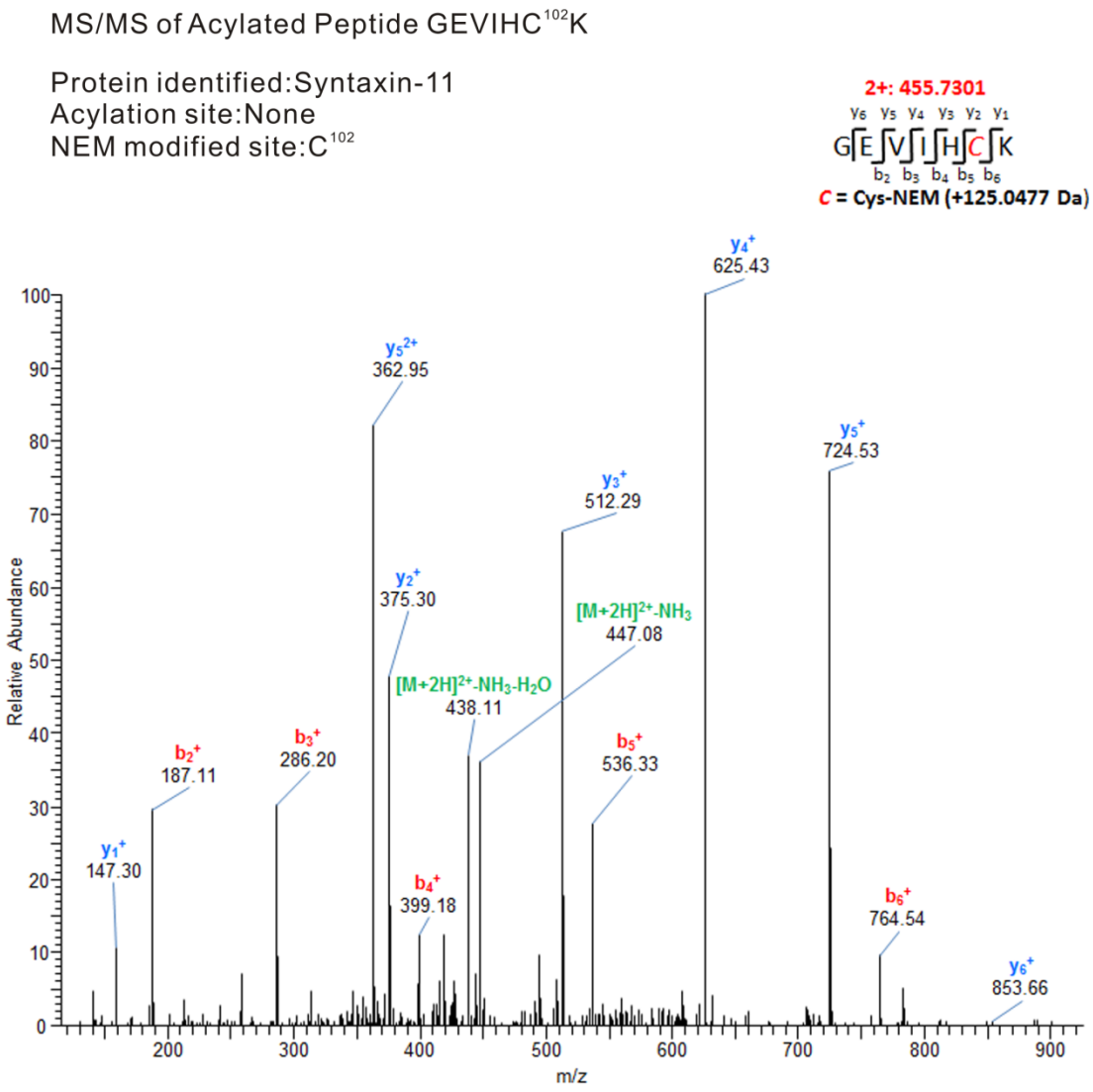


Figure 4-11 MS/MS of the NEM Modified Peptide from Syntaxin-11: GEVIHC^{102}K

Mass spectrums were shown with NEM modified for detecting acylation sites in peptide GEVIHC^{102}K of syntaxin-11. Modification sites were identified based on MS/MS fragmentation patterns. For clarity, only *y* and *b* ions are shown. The *y* ions are labeled in blue, the *b* ions in red.

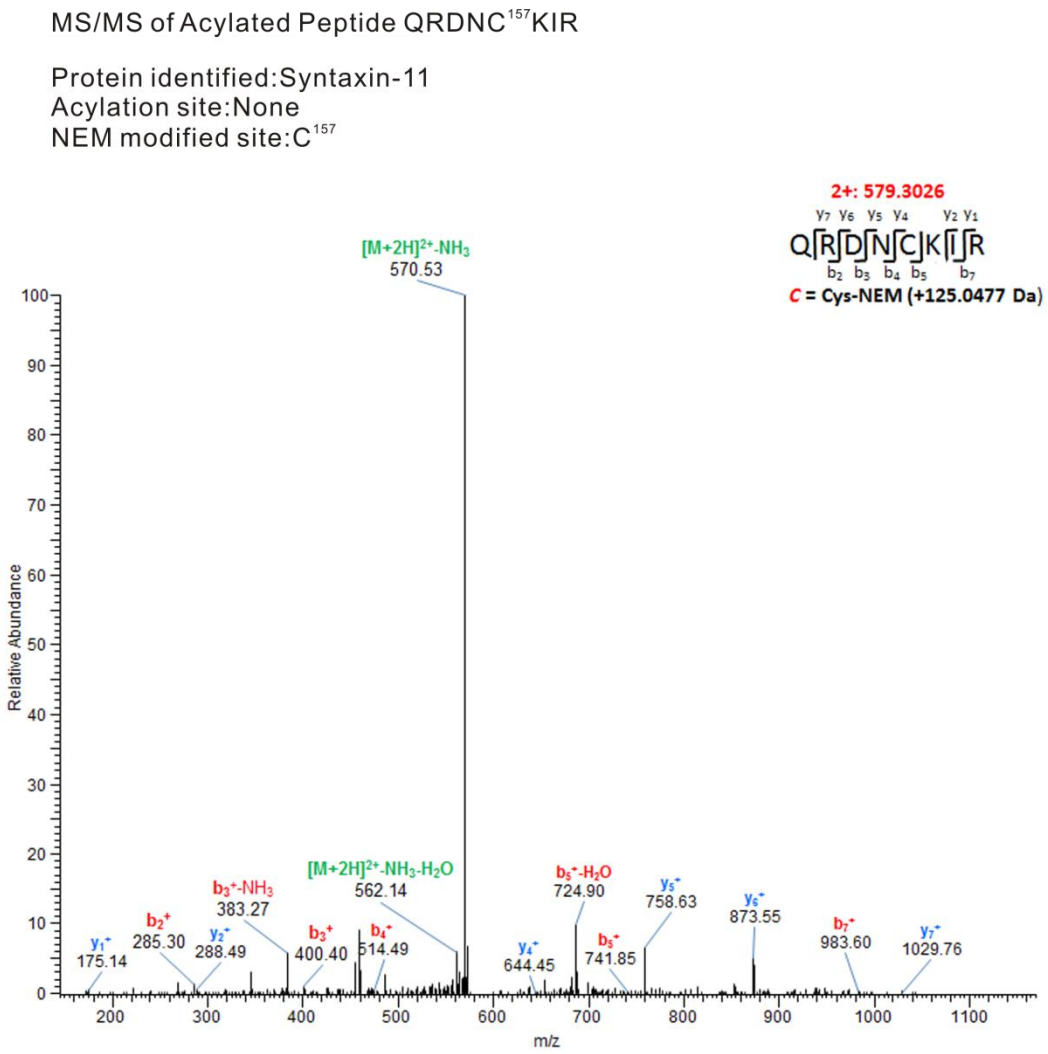


Figure 4-12 MS/MS of the NEM Modified Peptide from Syntaxin-11: QRDNC^{157}KIR

Mass spectrums were shown with NEM modified for detecting acylation sites in peptide QRDNC^{157}KIR of syntaxin-11. Modification sites were identified based on MS/MS fragmentation patterns. For clarity, only *y* and *b* ions are shown. The *y* ions are labeled in blue, the *b* ions in red.

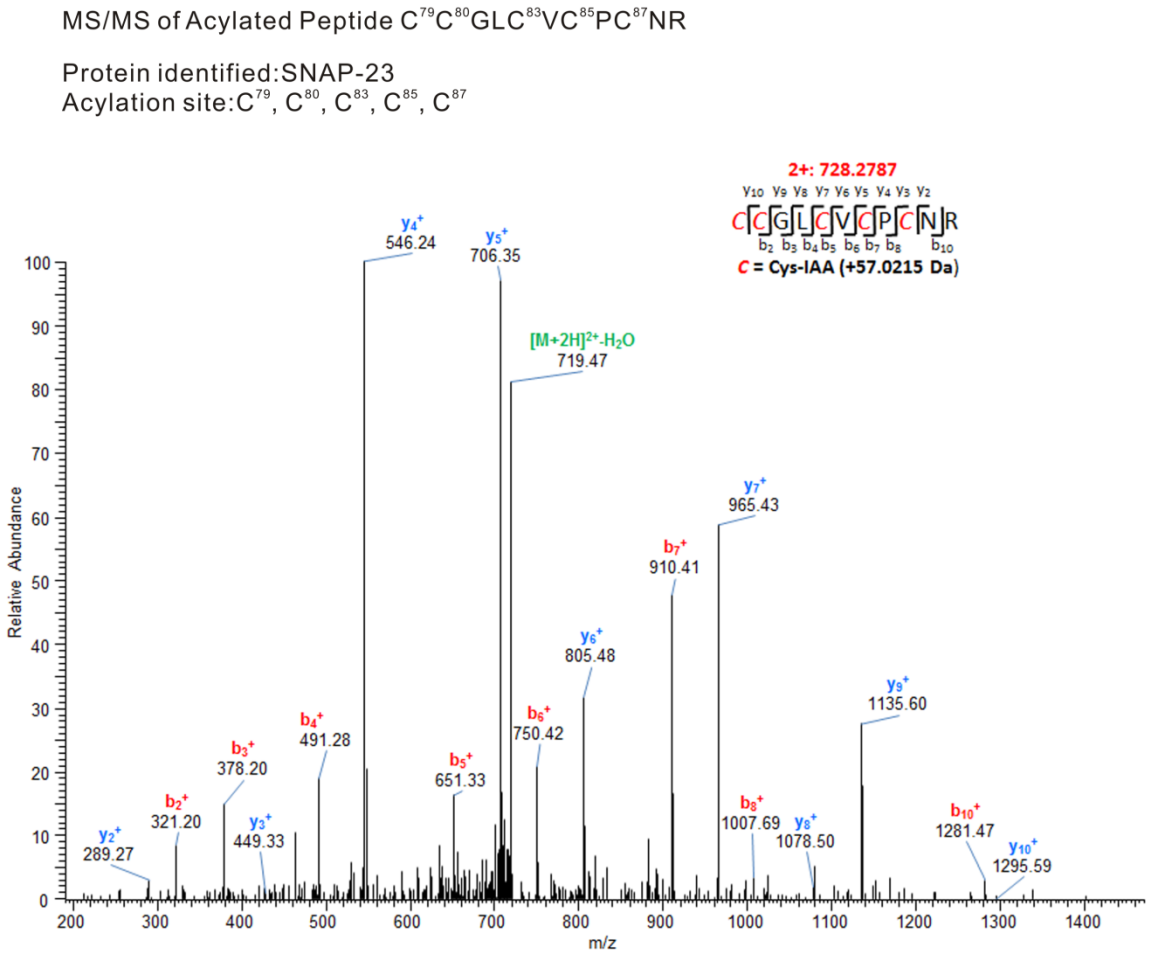


Figure 4-13 MS/MS of the Acylated Peptide from SNAP-23: $C^{79}C^{80}GLC^{83}VC^{85}PC^{87}NR$

Mass spectrums were shown with IAA modified for detecting acylation sites in acylated peptide $C^{79}C^{80}GLC^{83}VC^{85}PC^{87}NR$ of SNAP-23. Modification sites were identified based on MS/MS fragmentation patterns. For clarity, only *y* and *b* ions are shown. The *y* ions are labeled in blue, the *b* ions in red.

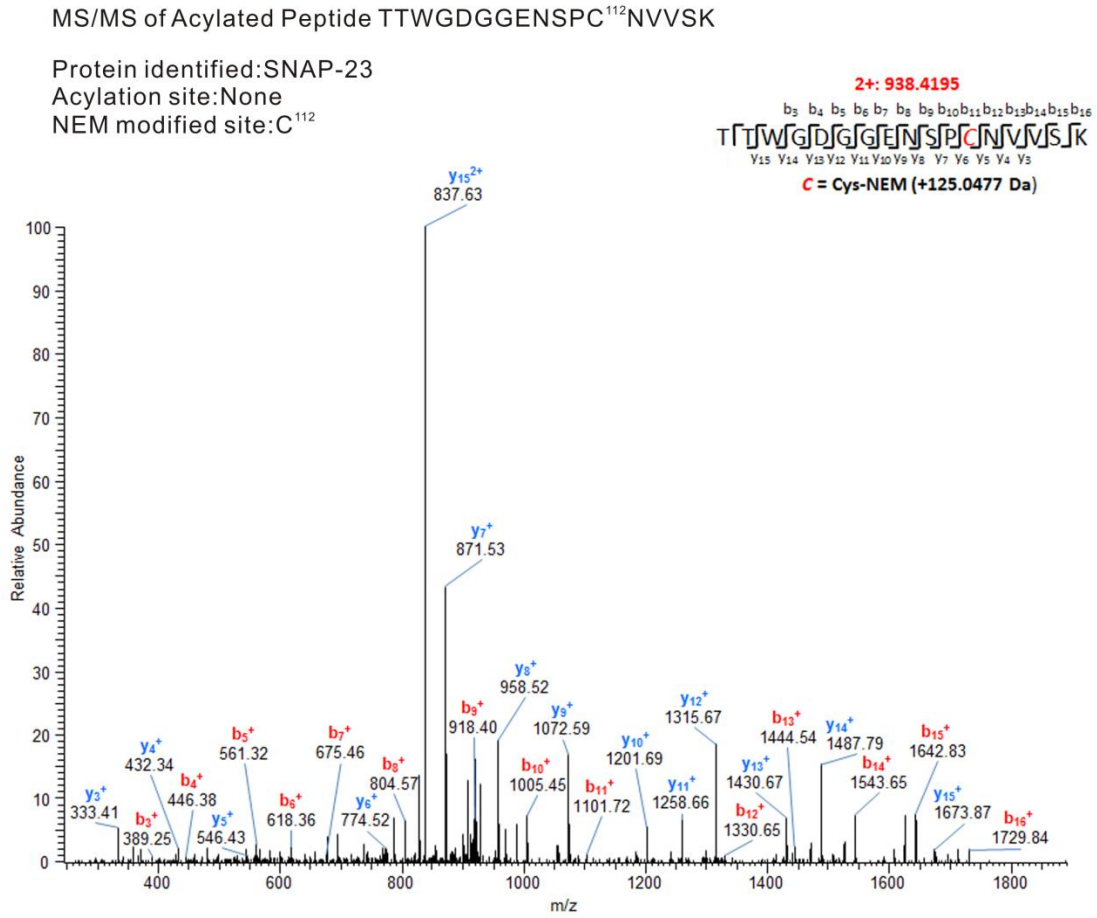
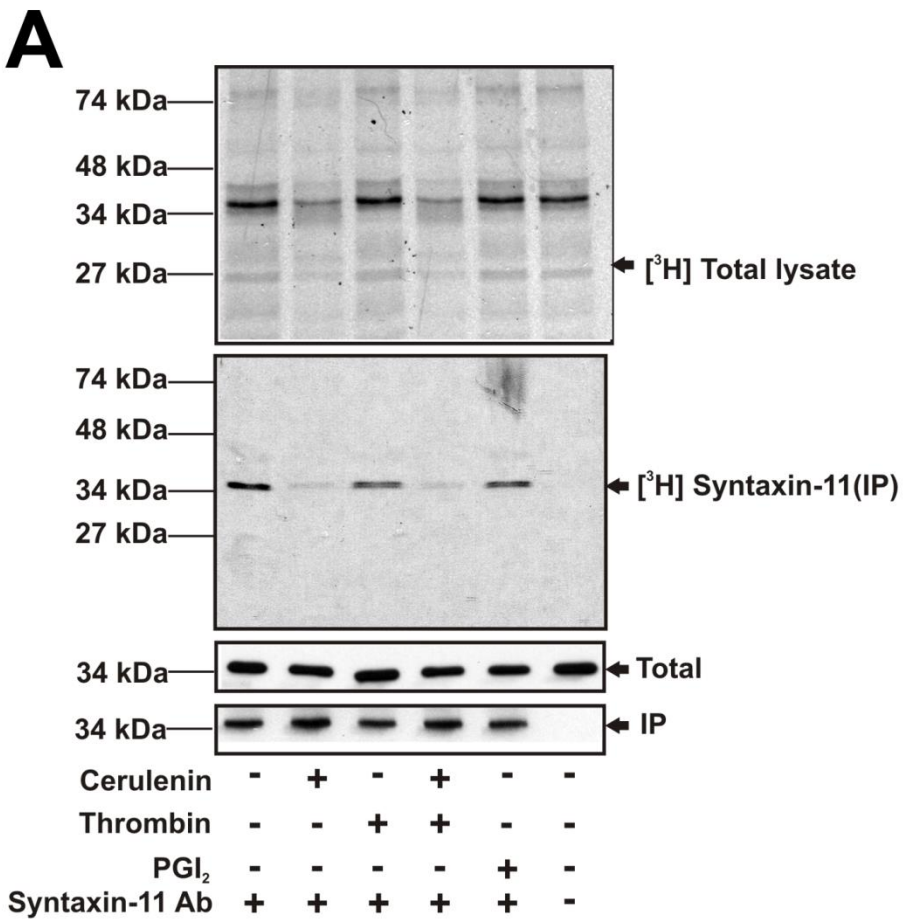


Figure 4-14 MS/MS of the Acylated Peptide from SNAP-23: TTWGDGGENSPC[112]NVVSK

Mass spectrums were shown with IAA modified for detecting acylation sites in acylated peptide TTWGDGGENSPC[112]NVVSK of SNAP-23. Modification sites were identified based on MS/MS fragmentation patterns. For clarity, only *y* and *b* ions are shown. The *y* ions are labeled in blue, the *b* ions in red.


A
74 kDa
48 kDa
34 kDa
27 kDa
[3H] Total lysate
74 kDa
48 kDa
34 kDa
27 kDa
[3H] Syntaxin-11(IP)
34 kDa
Total
34 kDa
IP
Cerulenin - + - + - -
Thrombin - - + + - -
PGI2 - - - - + -
Syntaxin-11 Ab + + + + + -

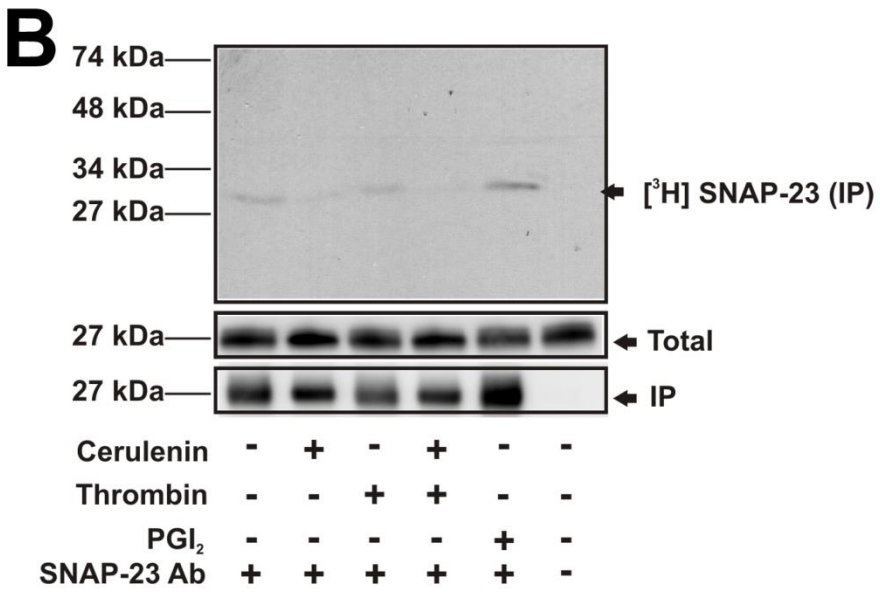

B
74 kDa
48 kDa
34 kDa
27 kDa
[3H] SNAP-23 (IP)
27 kDa
Total
27 kDa
IP
Cerulenin - + - + - -
Thrombin - - + + - -
PGI2 - - - - + -
SNAP-23 Ab + + + + + -

Figure 4-15 Dynamic Acylation of Syntaxin-11 and SNAP-23 in Platelets

Platelets were incubated with [^{3}H]-palmitic acid in the presence or absence of 500 μM cerulenin. Some were stimulated with thrombin and others were treated with PGI_2. Lysates were prepared for IP with anti-syntaxin-11 Ab (A) or anti-SNAP-23 Ab (B). Total protein (top) or immunoprecipitated proteins were separated by SDS-PAGE and visualized by autofluorography. Total protein and immunoprecipitated samples were also probed by western blotting with anti-syntaxin-11 Ab (middle) and anti-SNAP-23 Ab (bottom). Blots or data shown are representative of at least two independent experiments.

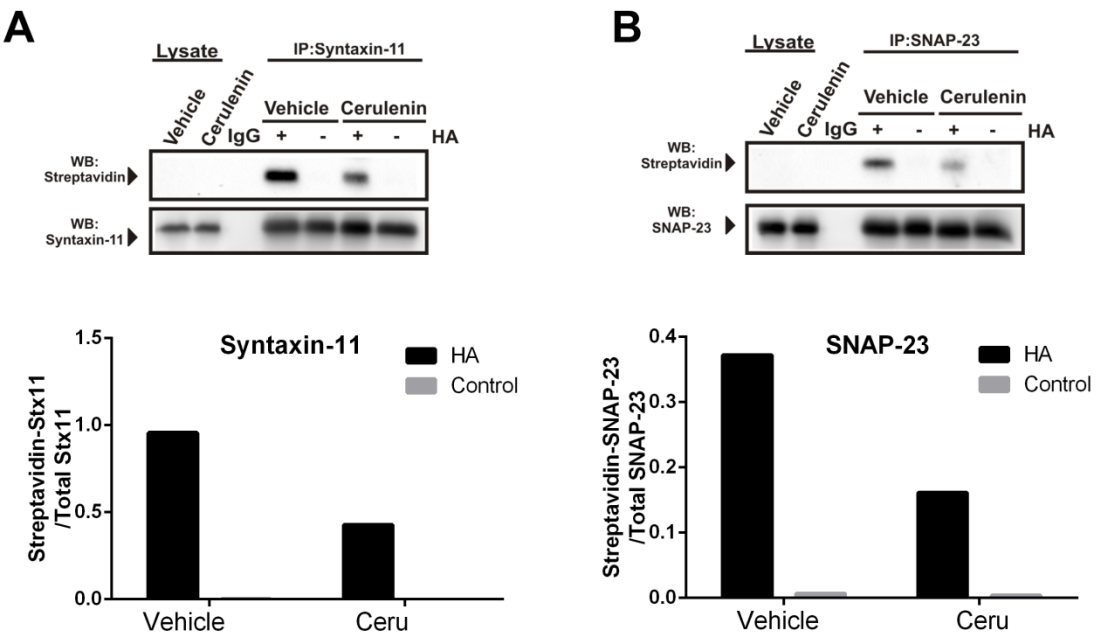


Figure 4-16 Cerulenin Inhibits Acylation of Platelet t-SNAREs

Platelets were treated with 500μM cerulenin (Ceru) for 2 hr. IP-ABE assay was performed for acylation detection after pre-incubation with cerulenin. The ratio of streptavidin-syntaxin11/total syntaxin-11 (A) quantification and streptavidin-SNAP-23/total SNAP-23 (B) quantification are shown. Blots or data shown are representative of at least two independent experiments.

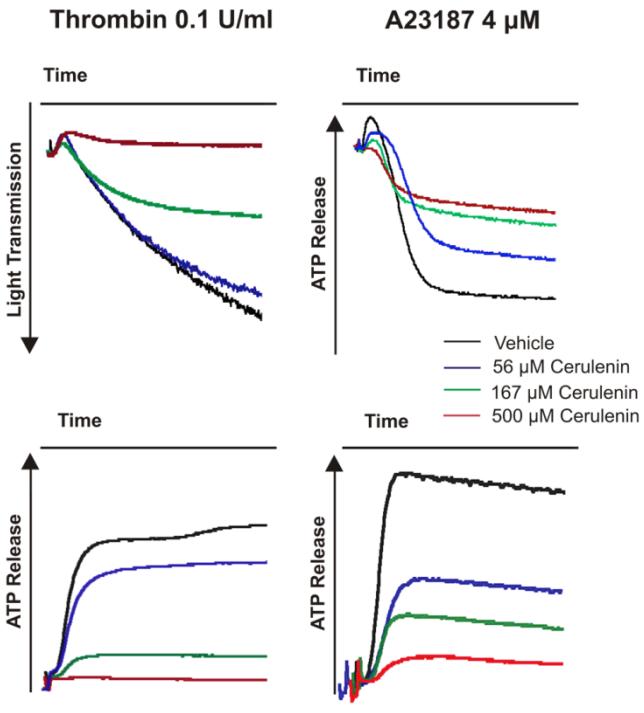


Figure 4-17 Cerulenin Inhibits ATP Release and Platelet Aggregation

Washed platelet suspensions (4×10^8/mL, 500 μL) were pre-treated with 56 μM, 167 μM and 500 μM cerulenin, respectively, for 2 hr at 37 ℃. Aggregation traces and ATP release were monitored. Tracings shown are representative of at least three separate experiments.

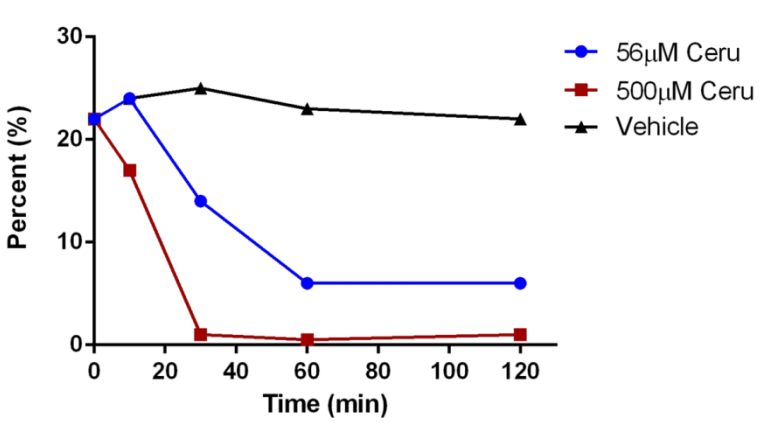


Figure 4-18 Time Dependent Inhibition of ATP Release after Cerulenin Treatment

Platelets were treated with cerulenin for the indicated times and aggregation in response to 0.1U/mL thrombin was measured by light transmission aggregometry. Data shown are representative of at least two independent experiments.

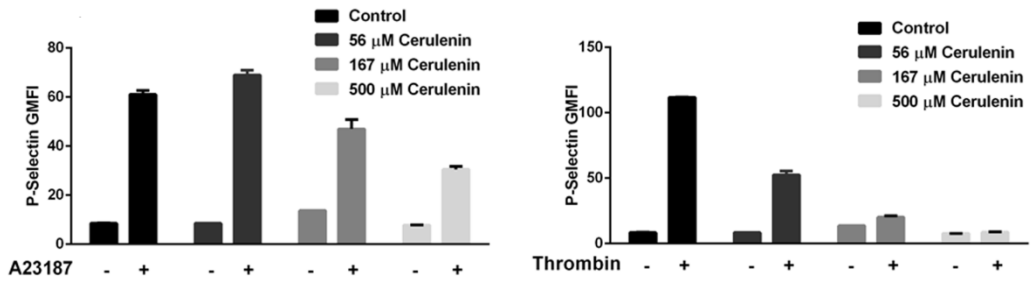


Figure 4-19 Cerulenin-Treatment Affects P-Selectin Exposure

Washed human platelets (1×10^8/mL) were treated with cerulenin (0, 56, 167, and 500μM) at 37°C for 2 hr. Thrombin (0.1 U/mL) or A23187 (4 μM) were used as agonists to stimulate platelets for 3 min. FITC-conjugated anti-P-selectin was added for 10 min. Fluorescent intensities were measured by FACS. The data were graphed using Geometric Mean Fluorescence Intensity (GMFI): P-selectin. Quantification of the P-selectin exposure is shown. Data shown are representative of at least three experiments.

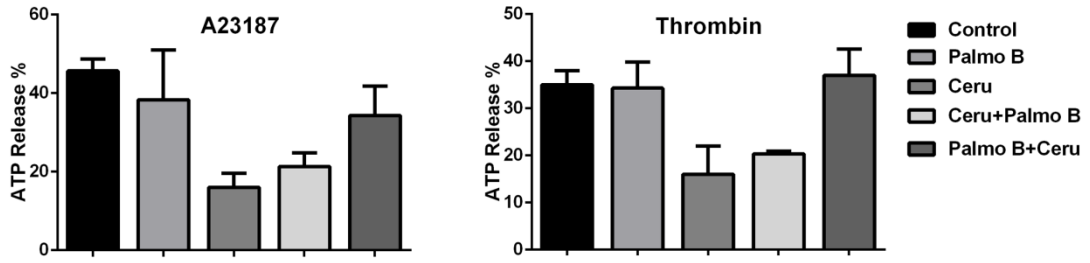


Figure 4-20 Palmostatin B Counteracts the Inhibitory Effects of Cerulenin

Washed platelet (4×10^8/mL) were treated with cerulenin (Ceru) or palmostatin B (Palmo B) alone ; or treated with both drugs sequentially: cerulenin or palmostatin B treated for 1 hr, the other drug treated for additional 1 hr. Buffer control group was also set up. Platelets were stimulated with agonists A23187 and Thrombin. ATP release was monitored. Quantifications of the ATP release in treated groups is shown (n=3). Data shown are representative of at least two experiments.

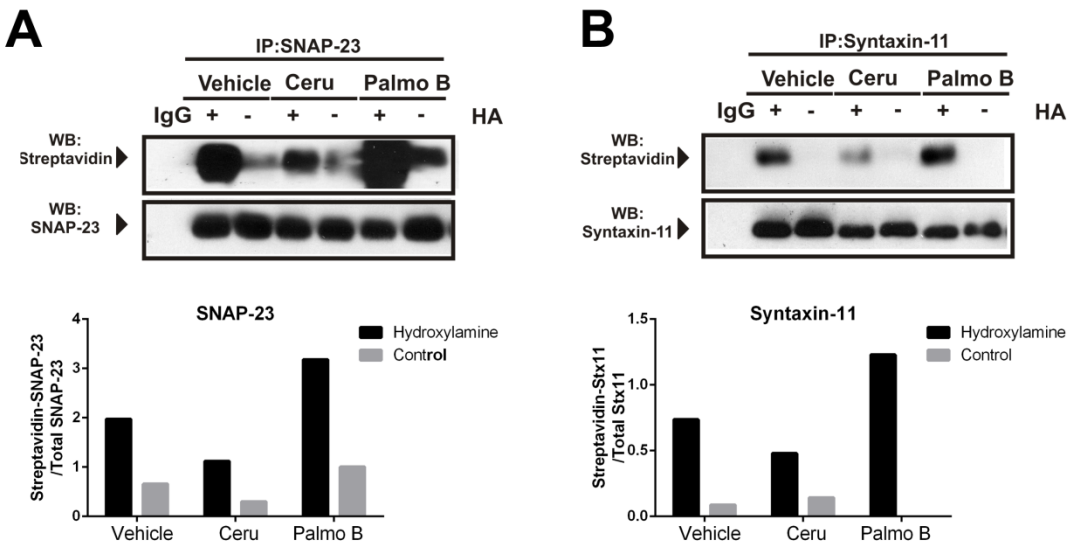


Figure 4-21 Acylation Accumulated in Palmostatin B-Treated Platelets

Washed platelets (1×10^9) were incubated in presence or absence of cerulenin (Ceru) and palmostatin B (Palmo B), and then lysed by lysis buffer. IP-ABE assay were used to detect acylation of syntaxin-11 and SNAP-23 after drug treatment. The ratio of streptavidin-syntaxin11/total syntaxin-11 (A) quantification and streptavidin-SNAP-23/total SNAP-23 (B) quantification are shown. Data shown are representative of at least two experiments.

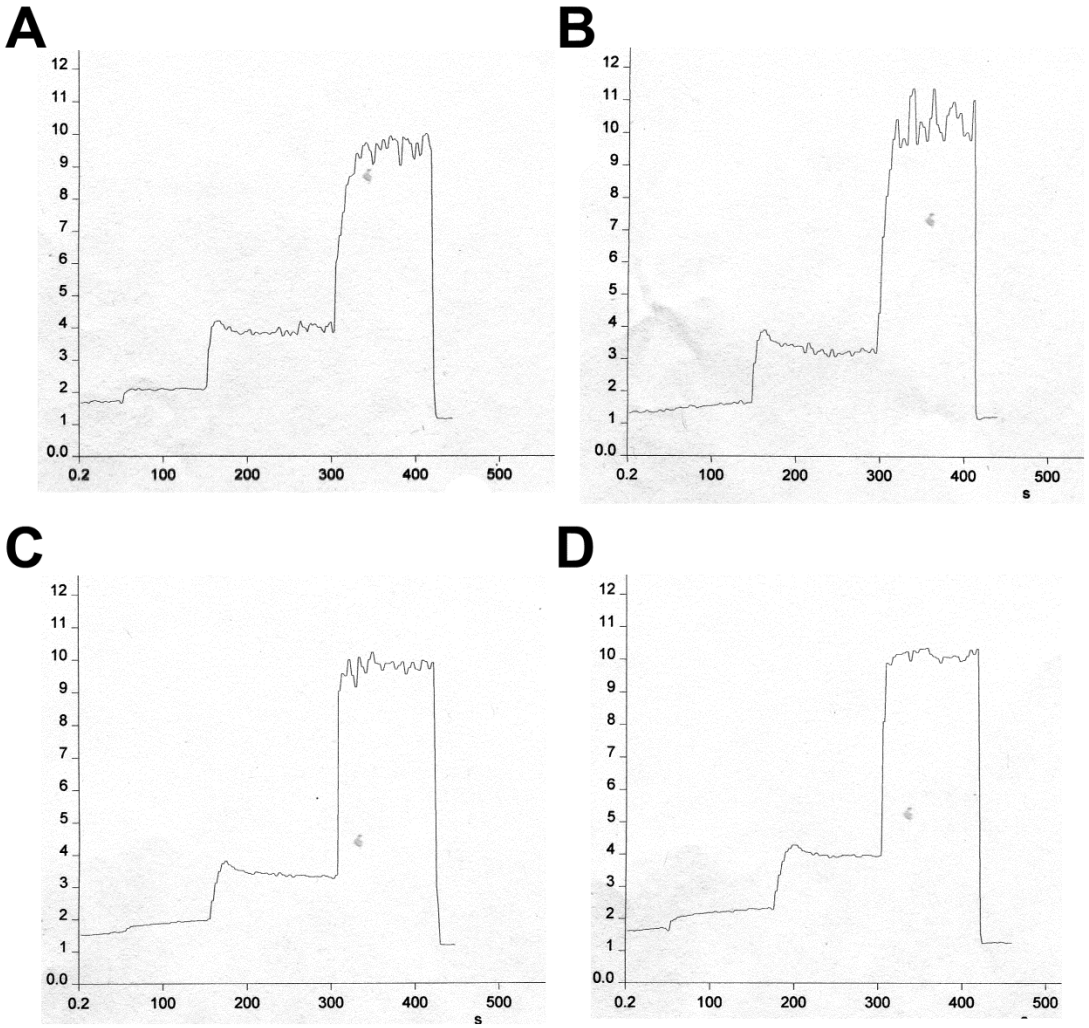

A
B
C
D
12
11
10
9
8
7
6
5
4
3
2
1
0.0
0.2
100
200
300
400
500
s

Figure 4-22 Intracellular Ca^{2+} Measurement in Cerulenin Treated Platelets

Intraplatelet calcium was measured using Fura-2-acetoxymethyl ester (Fura-2AM). Washed platelets (4×10^8/mL) in HEPES-Tyrode's buffer (pH7.4) were incubated in presence or absence of cerulenin at 37 ℃ for 1 hr. Then 1 μM Fura-2AM (Invitrogen) was added into platelets at 37 ℃ for 1 hr. After incubation, the Fura-2 loaded platelets were resuspended in HEPES-Tyrode's buffer (pH7.4). The platelet concentration was adjusted to 2×10^8/mL. 0.7 mM $CaCl_2$ and 750 μl platelets were added to siliconized cuvettes and stimulated with 0.1 U/mL thrombin and constant stirring. Fluorescence was analyzed by excitation at 340 nm and 380 nm, and emission was measured at 509 nm using a model LS55 Luminescence Spectrometer (Perkin-Elmer Cetus). The ratio of emissions was calculated simultaneously A) vehicle group, B) 56 μM cerulenin, C) 167 μM cerulenin, D) 500 μM cerulenin group. Data shown are representative of at least three experiments.

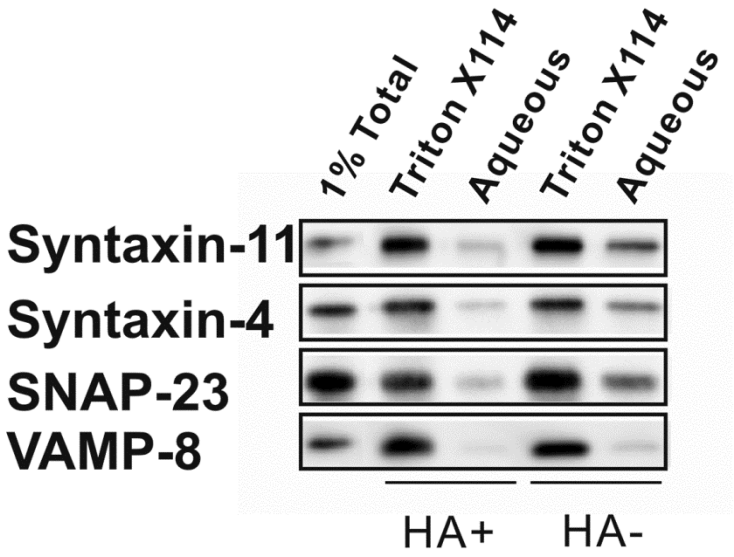


Figure 4-23 t-SNAREs Partition into Triton-114 Phase after HA Treatment

Platelet plasma membrane was solubilized in 2% Triton X-114 and treated with or without 0.3 M HA at 4℃ for 1 hr. After incubation, the samples were subjected to centrifugation and fractions were separated and collected. Triton X-114 fraction (detergent) and non-Triton X-114 fraction (aqueous) was subjected to western blot with anti-syntaxin-11, anti-SNAP-23, anti-VAMP-8, and anti-syntaxin-4 antibodies. Data shown are representative of at least two experiments.

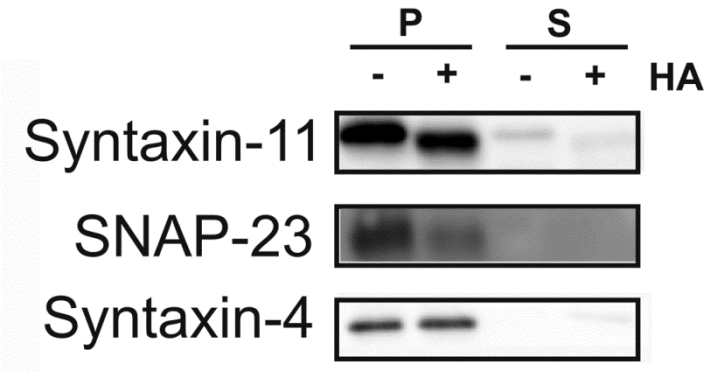


Figure 4-24 t-SNAREs are Not Released from Plasma Membranes by HA Treatment

Human plasma membrane were isolated and treated with or without 0.7M HA (pH 7.4) in buffer containing 1M NaCl for 30 min with rotating at 37 ℃. After incubation, ultracentrifuge was performed and supernatant and pellet were kept and subjected to western blotting. Blots are representative of 3 different experiments.

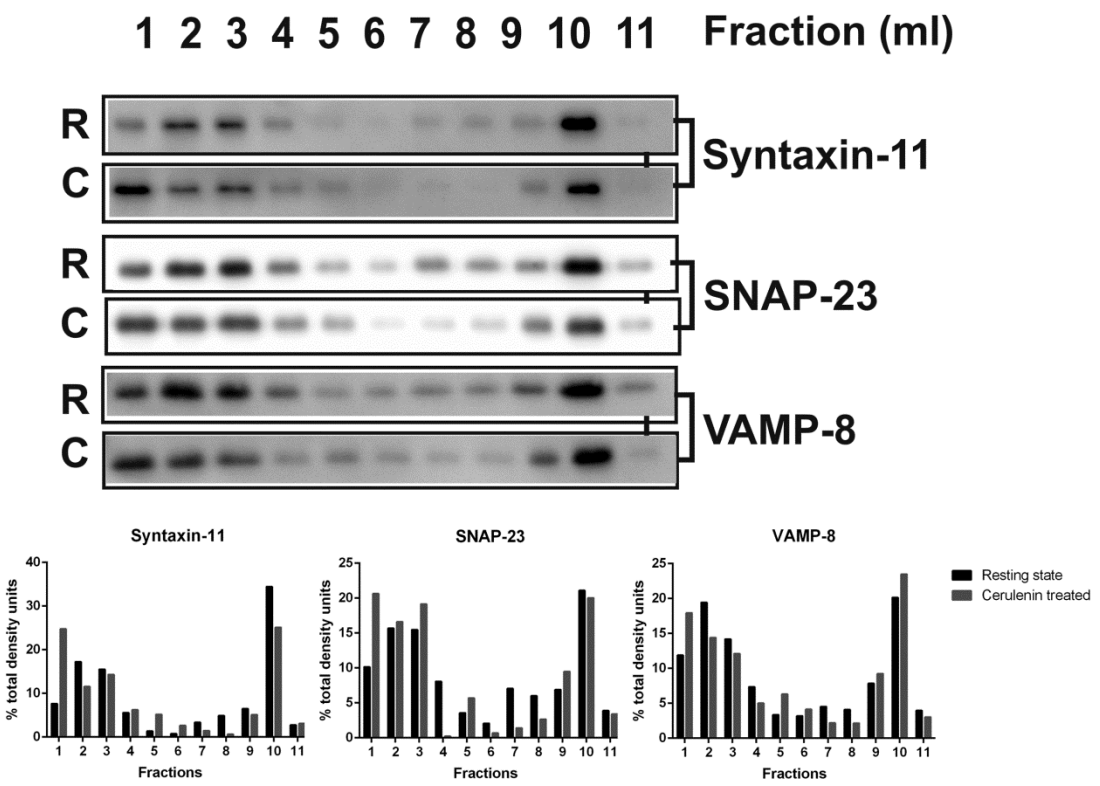


Figure 4-25 t-SNAREs are Present in Lipid Rafts Even after Cerulenin Treatment

Vehicle (R) or cerulenin treated (C) platelets were lysed in 2× raft lysis buffer and layered into ultracentrifuge tubes. The samples were subjected to ultracentrifugation at 200,000 × g for 18 hr at 4℃. The gradients were fractionated and probed for the indicated proteins by western blotting. Blots shown are representative of at least three experiments. Quantification of the specific proteins in each fraction is shown.

Chapter Five: Conclusions and Future Directions

Conclusions:

In this dissertation, I have studied post-translational modifications (PTM) in two platelet t-SNAREs to determine their dynamics and how they affect platelet function. In Chapter 3, using an *in vitro*, proteoliposome fusion assay, I demonstrated that purified IκB Kinase (IKK) phosphorylated SNAP-23 and this modification increased the initial rates of SNARE-mediated liposome fusion. Both phosphorylation and fusion enhancement required IKK activity. Mutants, containing phosphomimetics, Asp95 or Glu95, also showed enhanced initial fusion rates. In Chapter 4, I examined how the S-acylation of SNAP-23 and syntaxin-11 to understand how acylation may regulate the protein function and thus platelet exocytosis. Using metabolic labeling and hydroxylamine treatment, I showed that both contain thioester-linked acyl groups and that acylation dynamically turns over in platelets, despite their apparent lack of protein synthesis. Cerulenin pretreatment inhibited t-SNARE acylation and platelet function in a dose- and time-dependent manner while palmostatin B had no effect. Interestingly, pretreatment with palmostatin B blocked the inhibitory effects of cerulenin suggesting that maintaining the acylation state of platelet proteins is important for function. In summary, my work has shown that phosphorylation and acylation affect t-SNARE function and platelet secretion.

SNAP-23 Phosphorylation in Platelets

The Effect of SNAP-23 Phosphorylation on SNARE Complexes: In Chapter 3, I present data demonstrating SNAP-23 phosphorylation at Ser95 enhances membrane

fusion but is not required. Consistently, when Ser95 was changed to Asp or Glu, the mutant SNAP-23s also enhance membrane fusion *in vitro*. These data argue that SNAP-23 phosphorylation somehow affects SNARE function by: **1)** Promoting SNARE complex formation; **2)** Stabilizing SNARE complexes: and/or **3)** Affecting the spatial distribution of SNAREs. Given the inherent stability of SNARE complexes, as evidenced by their high melting points (193), it seems unlikely that stabilizing a SNARE complex, once formed, is the major role for phosphorylation. Our previous results support a role of SNAP-23 phosphorylation in SNARE complex formation. Functionally relevant SNARE complexes (SNAP-23/syntaxin-11/VAMP-8 or SNAP-23/syntaxin-2/VAMP-8) formed in activated platelets and could be detected by co-immunoprecipitation and western blotting (80). This corresponded to SNAP-23 phosphorylation; the SNAP-23 in the SNARE complex was phosphorylated on Ser95. Inhibition of or genetic deletion of IKKβ not only blocked platelet exocytosis and SNAP-23 phosphorylation, but also affected SNARE complex formation; no SNARE complexes were detected. While these data are suggestive of a mechanism, our understanding is incomplete. We must determine which form of SNAP-23 is the relevant IKKβ substrate in order to assemble the sequence of events and understand the molecular roles of SNAP-23 phosphorylation. Several forms of SNAP-23, in different protein complexes (SNAP-23, SNAP-23/syntaxin, SNAP-23/syntaxin/VAMP-8), are possible and each should be tested. If monomeric or heterodimeric SNAP-23 are preferred IKKβ substrates, then phosphorylation could drive association of the t-SNAREs with each other or with the v-SNAREs, or both.

This remains to be directly addressed experimentally, though the data in Chapter 3 does show that t-SNARE heterodimers can be phosphorylated *in vitro*.

In RBL-2H3, SNAP-23 associates with syntaxin-4/VAMP-2 and is phosphorylated upon stimulation (83). In this system, SNAP-23 is thought to recruit syntaxin-4 and VAMP-2 to lipid rafts and this recruitment correlates with SNAP-23 phosphorylation and the formation of phopho-SNAP-23/syntaxin-4/VAMP-2 complexes. These data suggest that SNAP-23 phosphorylation promotes SNARE complex formation in mast cells, as postulated in platelets and may also promote SNARE partitioning into lipid rafts. Given that SNAP-23 and syntaxin-11 are associated with lipids rafts (Figure 4-2), it would seem that SNAP-23 phosphorylation could have a similar effect in platelets as it does in mast cells. While this was examined in platelets, no conclusive data was generated and thus further examination of the relationship between phosphorylation, acylation, and raft association is warranted based on the data presented in this dissertation and that in the literature (83).

IKK Inhibitors and their Clinical Use: The IKK complex (IKKα/β/γ) is a ubiquitous regulator of NF-κB-based signaling in many cellular proceses. It has been shown to be involved in immune regulation, tumor growth/progression and several inflammatory diseases (194). IKK inhibitors do block SNAP-23 phosphorylation and IKK-enhanced membrane fusion (Figure 3-2 and Figure 3-3). They also block platelet exocytosis (80). IKK inhibitors, BMS 345541 and TPCA-1, used in this dissertation have not been widely used in clinical trials though there is some proof of their efficacy.

BMS 345541 promotes apoptosis in melanoma cells (195,196). TPCA-1 reduces tumor progression and increases the median survival time in a mouse tumor model (194). TPCA-1 also blocks the inflammation associated with arthritis and asthma. It decreases T cell proliferation and attenuates collagen-induced arthritis (197) as well as the inflammation in human smooth muscle airways and in a rat asthma model (198). There are several IKKβ inhibitors that have been developed in the recent years but few has been tested in pre-clinical trials (199-201). Our results show that IKKβ inhibition does block platelet secretion and significantly prolongs bleeding times in mice (80). The data in this dissertation further elucidates the mechanism by which IKKβ, through phosphorylation of SNAP-23, can regulate the membrane fusion needed for granule cargo release. Based on our data, IKKβ may be a viable therapeutic target for cardiovascular disease, and thus IKKβ inhibitors could be repurposed for cardiovascular disease treatment. Though this remains to be validated, it seems that bleeding could be a side-effect of IKKβ inhibition that will require monitoring in future clinical trials of the various IKKβ inhibitors that are being developed.

t-SNARE Acylation in Platelets

Enzymology of Platelet Acylation: Acylation/deacylation is dynamic and reversible, being regulated by synthetic PATs, substrate availability (*i.e.*, acyl-CoAs), and degradative palmitoyl thioesterases (Figure 5-1). Typical PATs contain a conserved cysteine-rich domain and an Asp-His-His-Cys (DHHC) motif (131-133) which serve

as family signatures. There are 25 ZDHHC proteins found in humans (125,135). PAT activities depend on these DHHC domains, as mutations of the cysteine abolish auto-acylation and acyl-transferase activity (136). Nine of the PATs are associated with human disease. The majority of PATs are associated with the endoplasmic reticulum (ER) or Golgi; however, a few localize to the plasma membrane (138). While it has been demonstrated that neuronal PATs exhibit distinct protein substrate specificity (139), the fact that there are several hundred acylated proteins in diverse cell types and tissues (112,202-205) suggests that at least some PATs must act on several substrates. Some PATs appear redundant and can compensate for each other. Thus several PATs could work together to control acylation of a specific protein. For instance, in neuronal cells, HIP14 (ZDHHC17) regulates acylation of SNAP-25 (131,140). However, coexpression of ZDHHC2 and SNAP-25 leads to enhanced palmitoylation of SNAP-25 in HEK293T (human embryonic kidney 293T) cells (141). When ZDHHC3, 7, 17 were co-transfected into HEK293T cells, they also increased palmitoylation of SNAP-25 (132). Acyl-CoAs might also be differentially used by PATs. Jennings *et al.*, found that ZDHHC2 and ZDHHC3 display different acyl-CoA specificities (206). ZDHHC2 used various acyl-coA species (C14,C16, C18, C20 and some unsaturated acyl-CoA) with almost equal efficacies; whereas ZDHHC3 displays much lower efficiencies with acyl-CoAs bearing longer carbon chains (C>16). The mechanism by which ZDHHCs recognize the different carbon lengths of the acyl-CoAs remain unknown. Overall, ZDHHCs may share some protein substrate specificity; but, these data strongly suggest that availability of enzymes as well as the

substrates (*i.e.*, acyl-CoA) may be very important in affecting the dynamics and extent to which a specific protein is acylated in a given cell type. At present, at least 13 ZDHHC enzymes or their mRNAs have been identified in platelets (Chapter 3, Table 1-1).

Currently, three dimensional structures of ZDHHC proteins are not available, so structure-based drug design is more difficult. Regulating acylation of proteins with the PAT inhibitors such as cerulenin or 2BP is perhaps not optimal due to their non-specificity. Many proteins are acylated and it is essential for protein function and cell growth. Non-selective acylation inhibitors may ultimately be quite cytotoxic. Blocking the activity of a specific ZDHHC protein may be less toxic and may also focus the study of acylation of certain proteins. Knockout mouse models can be used to determine which enzyme is responsible for regulating SNAP-23 and syntaxin-11 acylation. This would be helpful if specific enzymes are responsible for regulating acylation of t-SNARE and thus for platelet. However, given the potential redundancy of the various PATs and the fact that many have been detected in platelets directed PAT inhibition may not be a viable approach. Therapeutic titrations may however be a strategy for using PAT inhibitors as anti-thrombotics. Overall, current PAT inhibitors, *e.g.*, cerulenin and 2BP, are non-selective; they can block all of PATs. Identifying which PATs are responsible for regulating SNAP-23 and syntaxin-11 in platelets, it may provide novel targets to control the acylation of SNAP-23 and syntaxin-11 specifically but this will required more work (see discussion below).

Compared to the large number of acyltransferases-ZDHHC proteins, only 4

thioesterases have been identified in platelets: APT1, APT2, PPT1, and PPT2 (102,127,128). PPT1 and PPT2 are found in lysosomes and are important for degradation of acylated proteins (128). APT1 and APT2 are cytoplasmic enzymes and are thought to deacylate proteins non-specifically (167). From my work, palmostatin B inhibits the deacylation of both SNAP-23 and syntaxin-11 and thus counteracts the effects of cerulenin on platelet secretion We interpret this as proof that cycling of acylation occurs in platelets and is important for platelet function. This might suggest that palmostatin B could be repurposed to maintain platelet activity during storage; assuming that during storage, platelet acyl-CoA levels could decrease (or change composition) due to increased use to maintain platelet energetics or membrane integrity. The result could be a loss of t-SNARE acylation and thus a loss of secretory competency. Palmostatin B could delay the deacylation process and thus retard the loss of secretory competency. Future studies of stored platelets and the extent on t-SNARE acylation during storage will be required to address the feasibility of this approach.

Pharmacologic Acylation Effectors: PAT inhibitors, *e.g.*, cerulenin and 2BP, are widely used for inhibiting acylation. They affect acylation of a diverse set of proteins, *e.g.*, H-Ras and PSD95 (207,208). Both cerulenin and 2BP can non-selectively and irreversibly inhibit the activity of PATs. 2BP resembles palmitate (Figure 1-3) and was used as an acylation inhibitor *via* alkylating PATs. Because PAT inhibitors disrupt palmitoylation of diverse proteins including Ras, they can potentiality regulate cell proliferation, neural development and thus have been used trial for cancer treatment.

2BP impairs neural stem cell proliferation and induces cell apoptosis (209). 2BP inhibits the acylation of CD63 in platelets (159); however, in our experiments, 2BP (10μM-100μM) appeared to solubilize platelets, thus destroying them. Similarly, Jurkat T cells, are more sensitive to 2BP treatment and could not tolerate treatment overnight (160). From its chemical structure, 2BP contains a long chain fatty acid, which may act as a detergent to destabilize platelet membranes. Instead of 2BP, cerulenin was used in this dissertation to study acylation in platelets. Cerulenin is an antibiotic identified as a fatty acid synthetase inhibitor. Kawaguchi *et al.* showed that cerulenin reacts with the SH-group in the synthetase active site and thus inhibits fatty acid biosynthesis (210). Cerulenin has also been widely used as an acylation inhibitor *via* alkylating PATs (157). Similar to the results reported in this dissertation, it inhibited platelet secretion, aggregation, accumulation and delayed thrombi formation in vivo (211), indicating its potential as anti-thrombotic drug (102). From our results, platelet secretion was affected and inhibited by cerulenin through deacylation of SNAP-23 and syntaxin-11, which may be used in anti-thrombi formation and as a potential drug target. The mechanism of cerulenin as an acylation inhibitor is not well understood. Several studies showed that cerulenin can decrease the number of free thiols of either PAT or the substrate proteins (157,212). Besides 2BP and cerulenin, tunicamycin has been used to inhibit acylation, though it is also widely used to inhibit N-linked glycosylation (160). Because of its side effects, we did not use tunicamycin. Both 2BP and cerulenin also inhibit other enzymes involved in lipid metabolism. A novel PAT inhibitor, 2-(2-hydroxy-5-nitro-benzylidene) -benzo[b]thiophen-3-one

(Compound V), was tested and specifically inhibited all PATs reversibly by inhibiting autoacylation which is a different mechanism from either 2BP or cerulenin, which are irreversible inhibitors (207). Overall, most current acylation inhibitors are non-selectively and irreversibly inhibit the activity of PATs except Compound V, which is not commercially available. In the dissertation, cerulenin and 2BP have been chosen as "the best" acylation inhibitors available.

Palmostatin B inhibits APT and regulates protein deacylation, but not acylation. H- and N-Ras deacylation was inhibited by palmostatin B, thus affecting its distribution back to Golgi and most of Ras distribute in plasma membrane (213). Oncogenic-Ras-transformed MDCK-f3 cells reduced cell-cell contact and became multi-layers, thus oncogenic Ras activity contributes to cancer progression. Palmostatin B prevents the oncogenic transformation and induces a partial phenotypic reversion (214). In platelets, palmostatin B not only inhibits deacylation of t-SNAREs, syntaxin-11 and SNAP-23, but also counteract the effect of cerulenin (Figure 4-20). Due to non-selectivity, palmostatin B may be used for disrupting the cycle of acylation/deacylation. It may keep acylated protein in steady states and may be used to control platelet function. Neither PAT inhibitors nor palmostatin B are selective, which will be a challenge in clinical trials. How to improve or enhance the drug selectivity is worthy of future study. Either new specific drugs or drug delivery systems may solve the non-selective issues.

Effects of Acylation on Platelet SNAREs: Conventional SNARE proteins contain TMDs, which have been shown to be important in mediating membrane fusion (215).

It is unclear whether all lipid anchors can provide sufficient hydrophobicity to the SNAREs to mimic the TMDs. Acylation can provide the hydrophobicity needed for inserting into lipid bilayers, which helps SNAREs without TMD attach to membranes but what is sufficient for driving membrane fusion. Is the extent of acylation or the composition (*i.e.*, length and desaturation) of the fatty acids important for fusogenicity? SNARE proteins with lipid anchors can mediate membrane fusion in *in vitro* assays under specific conditions (216). Multiple lipid moieties can enable lipid-anchored SNARE to drive membrane fusion (217). Synaptobrevin-2 (VAMP-2), lacking a TMD, attached to proteoliposomes through one or two acylation sites is not sufficient for *in vitro* membrane fusion (218). The R-SNARE, Nyv1p, with an engineered lipid anchor that spanned half the lipid bilayer, also could not mediate membrane fusion in lipid mixing assay. However, addition of accessory proteins, such as HOPS, Sec17p, and Sec18p, did enable the lipid-anchored Nyv1p to drive membrane fusion (192). Studies by McNew *et al.* suggested that longer more hydrophobic lipid moieties could promote membrane fusion (219). TMDs were replaced by prenyl groups of different lengths and while C_{15} and C_{20} groups were insufficient for fusion, C_{55} groups could promote fusion *in vitro*. Overall, the length of lipid anchors, the number of modification sites, and the accessory proteins appear to affect efficiency of membrane fusion by SNAREs lacking TMDs. In my dissertation, syntaxin-11 and SNAP-23 lose their ability to drive membrane fusion, when platelets are treated with acylation inhibitors, but do not appear to lose their associations with membranes and lipid rafts. This suggests two related roles for SNARE acylation in platelets, dependent on the

extent of acylation. Membrane association and lipid raft distribution requires a lower level of acylation while membrane fusion requires a greater degree of acylation. One ramification of this conclusion is that as acylation decreases, the deacylated SNAREs could become inactive decoys for SNARE complex formation and, in fact, inhibit membrane fusion. Thus deacylation could negatively affect membrane fusion by reducing fusogenicity and by producing an inhibitory SNARE that diverts SNARE complex formation from fusogenic SNAREs.

Acylation Promotes SNARE Localization to Lipid Rafts: Lipid rafts are specialized cholesterol- and sphingolipid-enriched domains of plasma membrane. They are associated with several cellular functions including signaling and have recently been proposed to be important for efficient exocytosis (220). In my studies, syntaxin-11 and SNAP-23 are both enriched in lipid rafts upon activation. This is also seen in other cells such as NK cells and mast cells (80,99,121). Since SNARE complexes (syntaxin-11/SNAP-23/VAMP-8) formation increases upon activation (80) and VAMP-8 is enriched in rafts upon platelet activation, it is possible that SNARE complexes form in the rafts in an activation-dependent manner (Figure 4-2). Upon cholesterol depletion with MβCD, fewer SNAREs were present in lipid rafts and there was a decrease in stimulation-dependent release of ATP and less aggregation (Figure 4-4). Consistently in mast cells, ternary SNARE complexes (SNAP-23/syntaxin-4/VAMP-2) are redistributed into lipid rafts during exocytosis, and cholesterol-enriched, lipid rafts are important for exocytosis in mast cells (90). In PC12 cells, SNAP-23 contains 5 cysteines in its central cysteine-rich region, but it has

a three-fold higher affinity for lipid rafts than that of SNAP-25, which contain 4, suggesting that the extra cysteine provides one more acylation site which contributes to lipid raft association (120). In mast cells, phospho-SNAP-23 is also enriched in lipid rafts upon activation (99). While we have not confirmed a similar distribution for phospho-SNAP-23 in platelets, is seems possible that raft association may have several positive effects on SNAREs and SNARE complex formation by concentrating both SNAREs and SNARE-regulators. Together, acylation may play a positive role in the redistribution of proteins into lipid rafts in platelets. Whether lipid rafts are potential fusion sites for platelet exocytosis will require further experimentation.

Other Factors Affecting Raft Association: As discussed above, acylation aids protein localization to lipid rafts. Different acyl species may differentially affect this process. Given the turnover of acyl groups demonstrated in this dissertation it seems possible that dietary fatty acids could affect platelet function through their incorporation into the two t-SNAREs. Availability of fatty acids could also change the properties of the rafts through their incorporation into lipids. Lipid rafts are detergent–resistant membrane domains consisting of cholesterol and sphingolipids in outer leaflet. In the inner leaflet, there are mainly saturated fatty acyl chains (221). Polyunsaturated fatty acids (PUFAs) are also detected in lipid rafts (221). PUFAs from dietary sources, such as omega-3s, inhibit platelet aggregation and activation (222-224). Liang *et al.* showed that PUFA displaced Fyn protein from lipid rafts in Cos-1 and Jurkat cells, although it remained membrane-associated (225). Unsaturated fatty acids may cause displacement of proteins from rafts since it is difficult to pack

the bulky side chains into the ordered lipid rafts domains (226). Alternatively, Stulnig *et al.* showed that PUFA change the composition of lipid rafts, especially inner leaflets, causing displacement of acylation protein from membrane rafts. In these studies, PUFAs were incubated with Jurkat T cells and the concentration PUFA, in raft fractions, increased significantly. Palmitated proteins, Lck and LAT, were displaced from membrane rafts after PUFA treatment. The determining factor for acylated protein association with rafts appears to be due to altering raft composition, not protein acylation with PUFAs. Acylation with PUFA appears to have a lower turn-over rate compared to that of acylation with palmitate (225,227,228). It is unclear whether PUFA can change inner leaflet of lipid raft composition causing t-SNARE raft association or not. Whether PUFAs affect protein acylation and or alter membrane raft composition in platelets and further affects t-SNARE redistribution will required further experimentation.

Physiological Changes in Acylation: Both syntaxin-11 and SNAP-23 lack TMDs, but are acylated and, behave as integral membrane proteins. Lipid groups attached to cysteine-rich region of endogenous acylated proteins are diverse. While palmitate (C16:0) may be the major added fatty acid others, such as stearate (C18:0) and oleate (C18:1) could also be incorporated (229). The fatty acids released from other acylated platelet proteins have been analyzed by gas chromatography-mass spectrometry (230). The average compositions of lipids bound to proteins by thioester linkage are: 74% of palmitate, 22% stearate, and 4% oleate in inactivated platelets. When platelets were incubated with exogenous fatty acids, the lipid profiles changed, indicating that the

composition of extracellular lipids affects lipid profiles in platelets (230). Consistently, Fyn could be acylated with unsaturated fatty acids when Cos-1 cells were incubated with [^{3}H]oleic acid (18:1), or [^{3}H]arachidonic acid (20:4), but the efficiency of labeling is lower than that seen with [^{3}H] palmitic acid (16:0) (225). When platelets were incubated with 17-Octadecynoic acid (17-ODYA) for 2 hr, however, 17-ODYA incorporation into syntaxin-11 and SNAP-23 could not be detected (data not shown). Other fatty acids were not tested however it should be noted that incubating platelets with Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) did negatively affect [^{3}H] palmitic acid incorporation (data not shown) suggesting that they could be competitors for palmitoyl-CoA in the platelet Acyl-CoA pools Overall, there may be in the thioester-linked fatty acids attached to the platelet t-SNAREs, but further experiments will be required to determine if this heterogeneity is important for function. One step toward addressing this point is to determine the heterogeneity of acyl-CoA species present in platelets; this can be done mass spectrometry-based lipidomics.

Summary: The work presented in the dissertation show that the post-translational modifications, phosphorylation and acylation, are important for granule secretion in platelets. As primary t-SNARE, SNAP-23 can be phosphorylated and acylated at the same time. SNAP-23 phosphorylation at Ser95 enhances membrane fusion. It is the first data to directly show that IKKβ is the enzyme that controls SNAP-23 and affects SNARE complex formation. Together with our *in vivo* studies of IKKβ, the current work elucidates a picture that IKK is required for platelet secretion through SNAP-23

phosphorylation. IKK inhibitors may be repurposed for controlling platelet function and treatment of cardiovascular diseases. A similar tack may be possible with acylation. Inhibitors of acylation and deacylation were used to show that acylation is dynamic and essential for platelet function. Acylation of t-SNAREs is important for granule secretion. Palmostatin B, as a deacylation inhibitor, can keep acylation on and counteract the effect of cerulenin, which may be used for controlling platelet secretion. Due to the insufficient number of specific inhibitors, further pharmaceutical design is required to develop better inhibitors for regulating syntaxin-11 and SNAP-23. Overall, post-translational modification of SNAREs, together with regulatory proteins regulates granule secretion from several steps as shown in Figure 5-3, especially for the fusion step.

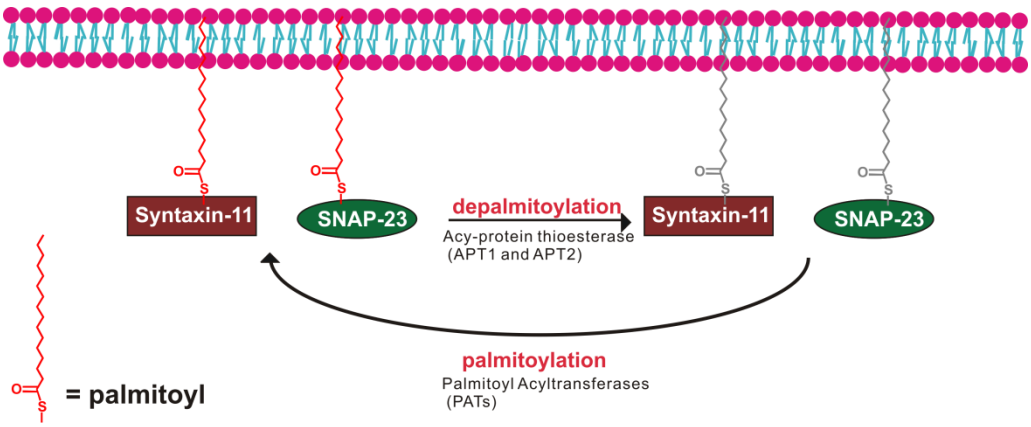


Figure 5-1 S-Acylation is a Reversible Process

S-acylation is a reversible process, regulated by acyl-protein thioesterase and palmitoyl acyltransferase respectively. In platelets, syntaxin-11 and SNAP-23 are two acylated proteins and essential for membrane fusion. Acylation state (red color labeled fatty acid) and deacylation state (grey color labeled fatty acid)

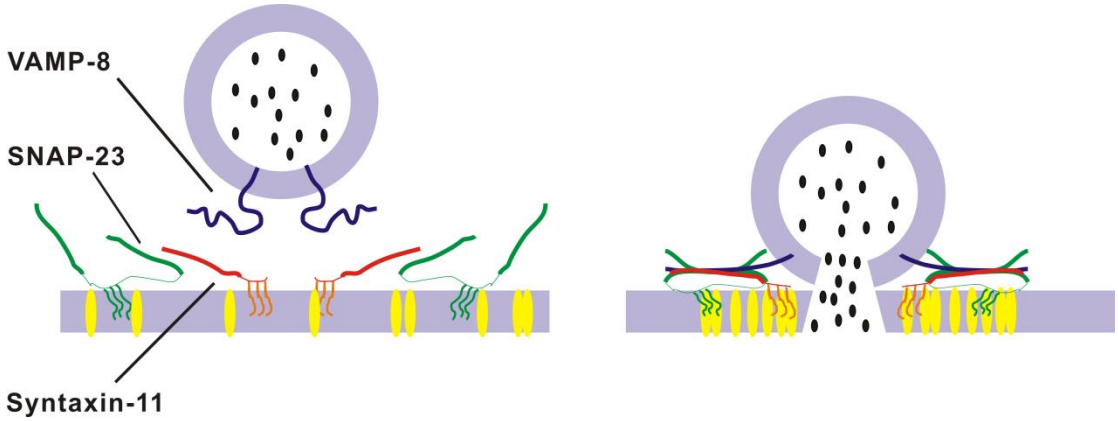


Figure 5-2 Acylation Affects the Role of t-SNARE in Platelet Secretion

Primary t-SNAREs, syntaxin-11/SNAP-23, lack of integral membrane domain and acylation modifications provide hydrophobic anchors and help them associate with plasma membrane. Acylation makes SNARE-mediated membrane fusion more effeciently and may localize it into lipid raft region upon platelet activation.

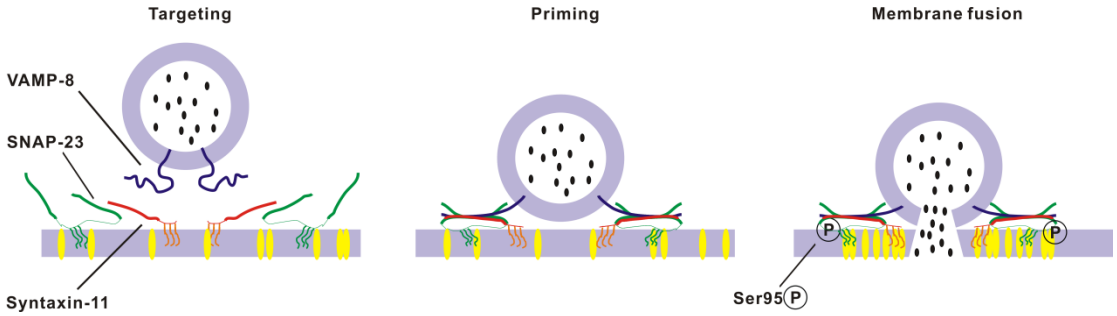


Figure 5-3 Steps in Platelet Granule Secretion

Secretory vesicles are targeted to active sites at the plasma membrane and subsequently they go through docking, priming, and fusion steps. During the process, these steps likely involve structural rearrangement between SNAREs and regulatory proteins. Phosphorylation of SNAP-23 may play a regulatory role in SNARE complex formation at steps. Acylation help t-SNAREs, lacking of TMD, to anchor to plasma membrane, and may also help them localize to lipid rafts for membrane fusion.

Appendices

ABBREVIATIONS

2BP	2-bromopalmitate
5-HT	Serotonin (5-hydroxytryptamine)
aa	Amino acids
ABE	Acyl-biotinyl exchange
ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
ApoE	Apolipoprotein E
APT	Acyl protein thioesterase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
Cerulenin	2,3 epoxy-4-oxo-7,10 dodecadienamide
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CMC	Critical micellar concentration
Compound V	2-(2-hydroxy-5-nitro-benzylidene)-benzo[*b*]thiophen-3-one
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DHHC	Asp-His-His-Cys
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence

EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FRET	Förster resonance energy transfer
GST	Glutathione S-transferase
FHL3	Familial hemophagocytic lymphohistiocytosis type 3
FHL4	Familial hemophagocytic lymphohistiocytosis type 4
FHL5	Familial hemophagocytic lymphohistiocytosis type 5
FITC	Fluorescein isothiocyanate
GMFI	Geometric mean fluorescence intensity
HA	Hydroxylamine
HeLa cells	Human epithelial cells
HRP	Horseradish peroxidase
IAA	Iodoacetamide
IKK	IκB kinase
IP	Immunoprecipitation
IP3	Inositol tri-phosphate

IPTG	Isopropyl-beta-D-thiogalactopyranoside
LAMP	Lysosomal associated membrane protein
LDL	Low density lipoprotein
MβCD	Methyl-β-cyclodextrin
NBD-PE	N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine
NK	Natural Killer
NRK	Normal rat kidney
OCS	Open canalicular system
PAGE	Polyacrylamide gel electrophoresis
PAS	Protein A-Sepharose 4
PAT	Protein acyltransferase
PC	1-palmitoyl-2-oleoyl phosphatidylcholine
PDGF	Platelet-derived growth factor
PE	1-palmitoyl-2-oleoylphosphatidyl-ethanolamine
PF4	Platelet factor IV
PGI_2	Prostaglandin I2
PI3P	Phosphatidyl inositol tri-phosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulphonylfluoride
PPT	Palmitoyl protein thioesterase

PRP	Platelet rich plasma
PS	1,2-dioleoyl phosphatidylserine
PVDF	Polyvinylidene fluoride
Rhodamine-PE	N-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine
RT	Room temperature
SNAP-23	Synaptosome-associated protein of 23 kDa
SNAP-25	Synaptosome-associated protein of 25 kDa
SNAP-29	Synaptosome-associated protein of 29 kDa
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
STXBP5/Tomosyn-1	Syntaxin binding protein 5
TCA	Tricholoracetic acid
TLR	Toll-like receptors
TMD	Transmembrane domain
VAMP	Vesicle associated membrane proteins
VEGF	Vascular endothelial growth factor
VWF	Von Willebrand factor
WT	Wild-type

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Member of American Association for the Advancement of Science (since 2011)

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Peer Reviewed Publications

1. **Jinchao Zhang**, Jing Chen, Haining Zhu and Sidney W. Whiteheart, Dynamic Cycling of t-SNARE Acylation is Important for Platelet Exocytosis (In preparation) 2016
2. Xiaobo Li, Meenakshi Banerjee, Shaojing Ye, Yunjie Huang, **Jinchao Zhang,** Susan S. Smyth, Andrew J. Morris, Sidney W. Whiteheart, Craig W. Vander Kooi, Direct interaction between Nrp1 and Integrin extracellular domains ***Biochemistry*** (Submitted) 2016
3. Michael C. Chicka , Qiansheng Ren, David Richards, Lance M. Hellman, Michael G. Fried, **Jinchao Zhang,** and Sidney W. Whiteheart, Role of Munc13-4 as a

Ca^{2+}-dependent tether during platelet secretion ***Biochemical Journal*** (in press) 2016

4.Shaojing Ye, Yunjie Huang, Smita Joshi, **Jinchao Zhang**, Fanmuyi Yang, Guoying Zhang, Susan S. Smyth, Zhenyu Li, Yoshimi Takai, and Sidney W. Whiteheart STXBP5/Tomosyn-1 is Critical for Platelet Secretion and Thrombosis. ***Journal of Clinical Investigation.*** Oct; 124(10):4517-28

5.Zubair A. Karim, **Jinchao Zhang**, Meenakshi Banerjee, Michael C. Chicka, Rania Al Hawas, Tara R. Hamilton, Paul A. Roche, and Sidney W. Whiteheart. I B Kinase (IKK) phosphorylation of SNAP-23 controls platelet secretion. ***Blood***. May 30, 2013; 121(22): 4567–4574.

6.Ying Cui, Liu Bai, **Jinchao Zhang**, Fuchun Yang, Sheng Li, Ying Zhao, Ying Gao. (2009) Establishment of assay for smooth muscle cell migration induced by PDGF in rat aorta. ***Journal of Dalian Medical University 31*** (3): 242-244. (Chinese)

Awards and Fellowships

2014 Max Steckler Fellowship， University of Kentucky

2014 Student Meeting Travel Support, University of Kentucky, USA

2012 "1st Place Poster Presentation Talk" Award at the 14th Biennial Midwest Platelet Conference, Cleveland, OH

2011-2012 Student Meeting Travel Support, University of Kentucky, USA

2005-2008 National Graduate Scholarship

2000-2005 Dalian Medical University Scholarship

Invited Talks

Jinchao Zhang 10/2012. Post-translational modifications of t-SNAREs control the platelet release reaction. 14th Biennial Midwest Platelet Conference, Cleveland, OH, USA.

Teaching Experience

Fall 2007 Biochemistry Course (Lab) for undergraduate Teaching Assistant

Spring 2011 Teaching Assistant in Biochemistry 401 FUNDAMENTALS OF BIOCHEMISTRY (undergraduate)

Fall 2012-Spring 2014 Mentoring Walker A. Reeves from MSTC at Paul Laurence Dunbar High School: 360 hour research project.

Training Program

2011 ASBMB Graduate and Postdoctoral Professional Development Program Washington, DC

2012 VASCULATA (training course in vascular biology) provided by North American Vascular Biology Organization (NAVBO), Vanderbilt University, Nashville, TN

Activities

Poster Judger for 8th Annual Postdoctoral Poster Session, College of Medicine, University of Kentucky, December 9, 2015

Chairperson of oral presentation sessions at Biochemistry, Departmental Retreat (2011)