Phosphorylation of SNAP-23 in Activated Human Platelets*

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Phosphorylation of SNARE proteins may provide a critical link between cell activation and secretory processes. Platelets contain all three members of the SNAP-23/25/29 gene family, but by comparison to brain tissue, SNAP-23 is the most highly enriched of these proteins in platelets. SNAP-23 function is required for exocytosis from platelet α, dense, and lysosomal granules. SNAP-23 was phosphorylated largely on serine residues in platelets activated with thrombin. Phosphorylation kinetics paralleled or preceded granule secretion. Inhibition studies suggested that SNAP-23 phosphorylation proceeds largely through a protein kinase C (PKC) mechanism and purified PKC directly phosphorylated recombinant (r-) SNAP-23 (up to 0.3 mol of phosphate/mol of protein). Five major tryptic phosphopeptides were identified in cellular SNAP-23 isolated from activated platelets; three phosphopeptides co-migrated with those identified in PKC-phosphorylated r-SNAP-23. In contrast, only one major phosphopeptide was identified when SNAP-23, engaged in a ternary SNARE complex, was phosphorylated by PKC. Ion trap mass spectrometry revealed that platelet SNAP-23 was phosphorylated at Ser23/Thr24 and Ser161, after cell activation by thrombin; these sites were also identified in PKC-phosphorylated r-SNAP-23. SNAP-23 mutants that mimic phosphorylation at Ser23/Thr24 inhibited syntaxin 4 interactions, whereas a phosphorylation mutant of Ser161 had only minor effects. Taken together these studies show that SNAP-23 is phosphorylated in platelets during cell activation through a PKC-related mechanism at two or more sites with kinetics that parallel or precede granule secretion. Because mutants that mimic SNAP-23 phosphorylation affect syntaxin 4 interactions, we hypothesize that SNAP-23 phosphorylation may be important for modulating SNARE-complex interactions during membrane trafficking and fusion.

SNAP1 receptor proteins (SNAREs) play a critical role in intracellular membrane trafficking/fusion in all eukaryotes (1, 2). SNAREs assemble into tight complexes that connect membranes and may induce membrane fusion. The prototypic SNARE complex contains one member from three different gene families: SNAP-25, syntaxin, and VAMP. The SNARE proteins contribute four α-helices to produce an extremely stable four-helix bundle with 16 highly conserved layers of interacting amino acid side chains (for classification of SNAREs and numbering the layers, see Refs. 3 and 4). The assembly and disassembly of the SNARE complexes are under the kinetic control of N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs), as well as Rab GTPases and Sec1/Munc18 family proteins (reviewed in Ref. 5).

SNAREs have been shown to be sites of phosphorylation at multiple sites with kinetics that parallel or precede granule secretion. Phosphorylation of SNARE machinery proteins may be an important signal in regulated secretion. Phosphorylation of synaptic vesicle proteins has been implicated in the regulation of neurotransmitter release (6). SNAPs have been shown to be substrates of purified or recombinant kinases in vitro (7–10), as well as of endogenous kinases in some neuroendocrine cells or yeast (11–14) (for review, see Ref. 15). Still, a clear picture has yet to emerge about the functional significance, the cell or developmental specificity, and the control of SNARE phosphorylation.

Platelets play an important role in thrombosis, atherosclerosis, and vascular remodeling through regulated secretion of effector molecules from platelet granules. The molecular secretory machinery in platelets has important homologies to the machinery found in neurons and other cells (reviewed in Ref. 16). Platelets contain SNAP-23 proteins (17–20) that form SNARE complexes in vitro that support SNAP-dependent NSF-ATPase activity (17). SNAP-dependent NSF is critical for exocytosis of α and dense granules (19), as well as lysosomes (21). Platelet membranes contain syntaxins 2 and 4, and they have been shown to be required for platelet secretion (18, 20–22). VAMPs have been shown to be present in platelets (20, 23, 24), and VAMP 3 and 8 are required for granule secretion (24). Platelets also contain SNAP-23, but less is known about SNAP-25 and SNAP-29. SNAP-23 is a ubiquitously expressed non-neuronal homolog (25) that shares 59% identity with SNAP-25 at the amino acid level. Like SNAP-25, SNAP-23 does not possess a transmembrane domain but it is palmitoylated on four-helix bundle with 16 highly conserved layers of interacting amino acid side chains (for classification of SNAREs and numbering the layers, see Refs. 3 and 4). The assembly and disassembly of the SNARE complexes are under the kinetic control of N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs), as well as Rab GTPases and Sec1/Munc18 family proteins (reviewed in Ref. 5).

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Regulated exocytosis or secretion from cells is a subset of intracellular membrane trafficking and fusion. In specialized secretory cells, such as neurons, neuroendocrine cells, or platelets, exocytosis from secretory vesicles is triggered by intracellular signals produced by cell activation through cell surface receptors or membrane depolarization. Phosphorylation of SNARE machinery proteins may be an important signal in regulated secretion. Phosphorylation of synaptic vesicle proteins has been implicated in the regulation of neurotransmitter release (6). SNAPs have been shown to be substrates of purified or recombinant kinases in vitro (7–10), as well as of endogenous kinases in some neuroendocrine cells or yeast (11–14) (for review, see Ref. 15). Still, a clear picture has yet to emerge about the functional significance, the cell or developmental specificity, and the control of SNARE phosphorylation.

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‡ The abbreviations used are: SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; NSF, N-ethylmaleimide-sensitive factor; VAMP, vesicle-associated membrane protein; PFP, platelet Sec1/Munc18 protein; PEC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase; PGI₂, prostaglandin I₂; MS/MS, tandem mass spectrometry; r-, recombinant; GST, glutathione S-transferase; FRP, platelet-rich plasma; BSA, bovine serum albumin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazineethanesulfonic acid; PVDF, polyvinylidene difluoride.

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With cell activation, SNAP-23 is phosphorylated at two sites in proteins were produced as described for SNAP-23 (28). Six recombinant phosphorilated members of this family and is required for secretion from all three types of platelet granules. Inhibition studies indicate that phosphorylation of SNAP-23 is PKC-dependent. With cell activation, SNAP-23 is phosphorylated at two sites in platelets, Ser23/Thr24 and Ser161. Both of these sites were also identified as phosphorylation sites when recombinant (r-) SNAP-23 was phosphorylated by purified PKC. Thus, SNAP-23 is a target for phosphorylation in platelets when cells are activated to secrete by thrombin. SNAP-23 phosphorylation kinetics parallel or precede granule secretion, suggesting that SNAP phosphorylation may play a functional role in exocytosis. This hypothesis was supported by the finding that mutants of SNAP-23 that mimic phosphorylation at Ser23/Thr24 showed reduced binding to syntaxin 4, indicating that phosphorylation of SNAP-23 at these sites may affect SNARE interactions.

EXPERIMENTAL PROCEDURES

Materials—Antibodies against human r-SNAP-23 were generated in rabbits and affinity-purified using immobilized antigens. Rabbit polyclonal anti-SNAP-23 peptide (amino acids 192–216) antisera were from Synaptic Systems (Gottingen, Germany). Anti-syntaxin 4 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine monoclonal antibodies, 4G10, and purified PKC were from Upstate Biotechnology (Lake Placid, NY); anti-P-selectin monoclonal antibodies (AK-R) were from Biozide (Kennebunk, ME). Monoclonal antibodies against human recombinant SNAP-23 were generated in mice. All kinase inhibitors and recombinant PKC isozymes were from Calbiochem (San Diego, CA). 5-Hydroxy-2'-deoxycreatinine sulfate (1(3)C)serotonin was from Amersham Biosciences.

Preparation of Recombinant SNAREs and SNARE Complexes—SNAP-23 and the cytosolic domain of syntaxin 4 were produced as described (28). SNAP-23 Ser23→Asp/Thr24→Asp and Ser161→Asp mutants were produced by overlap PCR using the following primers: for Ser23→Asp/Thr24→Asp, CCATGGATAATCTGTCATCAGA and CTAAACCGGATTCTC, GATGGACTGCTTGAAGATGAGTTGAAGATTGGTTAG and CGATCCTAATGCTGCAATGTTTG, CCATGGGATCATCAAG and CGATCCTGATCTGCTGCATATGTCATAGTTC; for Ser161→Asp, CCATGGATAATCTGTCATCAGA and CTAAACCGGATTCTC, GATGGACTGCTTGAAGATGAGTTGAAGATTGGTTAG and CGATCCTAATGCTGCAATGTTTG, CCATGGGATCATCAAG and CGATCCTGATCTGCTGCATATGTCATAGTTC; for Ser23→Ser161→Asp, CCATGGATAATCTGTCATCAGA and CTAAACCGGATTCTC, GATGGACTGCTTGAAGATGAGTTGAAGATTGGTTAG and CGATCCTAATGCTGCAATGTTTG, CCATGGGATCATCAAG and CGATCCTGATCTGCTGCATATGTCATAGTTC. DNA from two clones each for Ser23→Asp/Thr24→Asp and Ser161→Asp mutants were sequenced (verified by double-stranded DNA sequencing) were ligated in-frame into the NcoI and KpnI sites of pProEX HTA expression vector (Invitrogen). Recombinant SNAP-23 proteins were produced as described for SNAP-23 (28). Six recombinant GST-tagged proteins representing the native N- and C-terminal SNARE motifs of SNAP-23 as well as the Asp or Ala mutants in positions Ser23→Asp/Thr24→Asp and Ser161→Asp were also produced using standard protocols (29) and the GST tag was cleaved with thrombin. The cytosolic domain of human VAMP 2 was produced as recombinant GST-tagged (Amersham Biosciences) protein in Esherichia coli using standard protocols (29), the GST tag was cleaved with thrombin, and VAMP 2 was further purified by FPLC as described (30). Ternary complexes of recombinant SNAP-23, syntaxin 4, and VAMP 2 were produced and purified as described (31).

Binding Assays—The binding of SNAP-23 and SNAP-23 mutants to syntaxin 4 was studied in a solid-phase assay similar as described previously (18, 28). Wells of a microtiter plate were coated with recombinant syntaxin 4 (50 μl of 10 μg/ml) for 1–2 h and blocked with 2% bovine serum albumin. Coated syntaxin 4 was incubated with native or mutated SNAP-23 (using the indicated concentrations, time, and temperatures). The wells were washed to remove unbound proteins, and polyclonal anti-SNAP-23 antisera (200-fold dilution) was added for 1 h. (Preliminary experiments confirmed that the SNAP-23 antibody bound equally well to the SNAP-23 point mutants). After washing, the bound antibody was detected by 125I-protein A (50,000 cpm/50 μl). The samples were heated in a 37 °C water bath; a small portion of the sample was used to measure the protein concentration. P-selectin expression on non-stimulated cells was <2% of that in thrombin-stimulated cells, confirming that the platelets were not activated. The samples were combined and diluted with buffer to 1.4 × 109 platelets/ml of suspension. Half of the platelet sample was activated with 1 unit/ml thrombin, whereas the other half was treated with 1 μg prostaglandin I2 (PGI2) for 30 s. Platelets were then solubilized by adding 0.1 volume of lysis buffer (2.5% SDS, 50 mM EDTA, 100 mM NaVO4, 100 mM NaF, 1 mg/ml leupeptin) and 0.05 volume of freshly dissolved 20 mM phenylmethylsulfonyl fluoride, and samples were put on ice. The samples were boiled in 1-mL aliquots for 5 min, allowed to cool to room temperature, and diluted with 4 volumes of 1.25× buffer A (buffer A: 1% Triton X-100, 50 mM EDTA, 5 mM NaVO4, 5 mM NaF, 100 μg/ml leupeptin, 100 μg/ml aprotinin, 20 mM Tris-HCl, pH 7.4). Anti-SNAP-23 antibodies (affinity-purified using immobilized SNAP-23) were coupled to cyanogen bromide-activated Sepharose 4B (Sigma). The gel (1 mL of settled gel, 4 mg of coupled IgG/ml) was equilibrated with buffer A before adding it to the platelet lysate. After a 2-h rotation at room temperature, the gel was settled in a small column and washed first with 10 ml of buffer A followed by 40 ml of 140 mM NaCl, 20 mM Tris-HCl, pH 7.4. Bound proteins were eluted with 5 ml of 20 mM CHAPS, 0.1 mM glycine, pH 2.9. The pH was neutralized with 50 μl of 3 M Tris, and the sample was concentrated to 100 μl using Centricon 10 (Amicon, Beverly, MA) concentrator. The concentrated sample contained ~4 μg of SNAP-23 as determined by quantitative immunoblotting with anti-SNAP-23 antibodies obtained with human r-SNAP-23. After reducing SDS-PAGE (12% gel) and visualizing with traditional Coomassie staining, the band corresponding to SNAP-23 was subjected to in gel reduction, carboxymethylation, and tryptic digestion (Promega). Phosphorylated peptide sequences were determined using a 75-μm reverse phase microcapillary (AceGlass) system with a double-electrostatically focused quadrupole ion trap repetitively surveyed the range m/z 395–1600, executing data-dependent tandem mass spectrometry (MS/MS) for peptide sequence information on the four most abundant ions in each survey scan. Phosphopeptides were acquired with a relative collision energy of 30% and an isolation width of 2.5 daltons, and recurring ions were dynamically excluded. After data base correlation with the algorithm SEQUEST (34), phosphorylated peptides were confirmed by manual, de novo interpretation of the MS/MS spectra using Fuzzylens (35).
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Phosphorylation of the Effects of Kinase Inhibitors on Thrombin-induced SNAP-23 Phosphorylation in Platelets—Thrombin-induced SNAP-23 phosphorylation in intact (not permeabilized) platelets was measured as described for syntaxin 4 phosphorylation (28) with the modification that the 32P-loaded platelets were incubated with the kinase inhibitors at room temperature for 30 min instead of at 30 °C for 15 min. Measurement of Granule Secretion and SNAP-23 Phosphorylation in Parallel Assays—Human PRP was prepared from freshly drawn blood as described (9). FGL (0.2 μM) was added, and the PRP was incubated in a 37 °C water bath for 1 h with and without [14C]serotonin addition (3 μl of 50 Ci/ml, 57 mCi/mmol [14C]serotonin per ml of PRP). Platelets were pelleted with centrifugation and resuspended in 0.33 times the volume of the original PRP in a buffer containing 157 mM NaCl, 2.8 mM KCl, 1 mM MgCl2·6H2O, 5.5 mM glucose, 2.5 mM EDTA, 0.2 mM PEG, 10 mM Hepes, pH 7.4. Then 0.1 volume of water or 32P-labeled inorganic phosphate (10 mCi/ml, specific activity of 285 Ci/mg) was added. The samples were incubated in a 30 °C water bath for 1 h. Then 0.01 volume of 10% bovine serum albumin (BSA) was added, and the platelets were pelleted by centrifugation and resuspended in the above buffer now containing 0.1% BSA and no PEG. Platelets loaded with [14C]serotonin or 32P were used to measure granule secretion or phosphorylation of SNAP-23, respectively. Non-stirred platelet samples were activated with various concentrations of thrombin at room temperature (22 ± 1.5 °C).

For granule secretion measurements, platelet activation was terminated by adding 1 volume of 2% ice-cold paraformaldehyde and putting the samples on ice. a granule secretion was monitored by measuring P-selectin expression with phycoerythrin-conjugated anti-CD62 antibodies AC1.2 (Becton Dickinson) and flow cytometry (FACSCalibur, Becton Dickinson) as described (36). Typically, 2.5 μl of fixed platelets were added to 97.5 μl of antibody solution. After 15 min the samples were diluted with 1 ml of Tyrode's buffer containing 0.35% BSA and analyzed. Total or 100% P-selectin expression was defined as that induced in 10 min by 1 unit/ml thrombin. P-selectin expression on non-stimulated platelets was <2%. The remaining samples were centrifuged at 2500 × g for 1 min, and the supernatants were used for scintillation counting of [14C]serotonin to assess dense granule secretion and for the measurement of metabolic ATP pool in the cytosol. For earlier reports. For example, P-selectin was significantly more abundant in the α granule fraction than the plasma membrane fraction (Fig. 1A). SNAP-23 was readily detected in platelet samples containing as little as 1.5 μg of protein, but only a very faint band was seen in the brain lysate samples containing 20-fold more protein. In contrast, SNAP-25 (which migrated faster than SNAP-23) was expressed robustly in the brain and only trace amounts were detected in the platelet samples. SNAP-29 was detected as a single band in the brain sample with a relative mass of ~33 kDa. Two major bands of slightly larger relative mass, 34–35 kDa, were seen in the platelet that were not detected when the primary antibody was omitted (data not shown). Platelet and brain SNAP-29 proteins may differ because of alternate transcript splicing or post-translational modification. These data indicate that SNAP-23 is a major SNAP-23/25/29 family protein in the platelet.

SNAP-23, similar to SNAP-25, is anchored to membranes through palmitoylated cysteine residues (26). A recent report suggested that platelets contained a significant pool of soluble, not membrane-associated, SNAP-23 (22). To examine the subcellular localization of platelet SNAP-23, platelets were disrupted by nitrogen cavitation and subcellular fractions were separated by sucrose gradient ultracentrifugation according to the method of Broekman (38). Fig. 1B shows that SNAP-23 was found almost exclusively in the plasma membrane fraction. At high sensitivity levels, the chemiluminescence images showed small amount of SNAP-23 in the α granule fraction but not in the cytosome (data not shown). In these experiments, the subcellular locations of other platelet proteins were in agreement with earlier reports. For example, P-selectin was significantly more abundant in the α granule fraction than the plasma membrane fraction (Fig. 1B), confirming that non-stimulated platelets were analyzed. Platelet Munc18 was detected in both the cytosome and the plasma membrane, and to a lesser extent in α and dense granule fractions. Platelet syntaxin 4 was found mainly in the plasma membrane, but a small portion of it could be detected in the α granule fraction.

Inhibition experiments were performed to determine whether SNAP-23 played a role in Ca2+-triggered granule exocytosis. Anti-SNAP-23 antibodies inhibited nearly all secretion from α, dense, and lysosome granules in streptolysin O-permeabilized platelets (Fig. 1C). These data are in line with earlier reports (21, 22, 39) and demonstrate the functional importance of SNAP-23 in the mechanism of regulated exocytosis in the platelet.

SNAP-23 Is Phosphorylated in Thrombin-activated Human Platelets—To further dissect the molecular mechanism of regulated exocytosis, we examined the phosphorylation of SNAP-23 in activated human platelets. For these studies, the metabolic ATP pool in the cytosome was labeled with 32P-labeled inorganic phosphate. Non-stimulated, thrombin-activated, or PGL2-passivated platelets were lysed, and phospho-SNAP-23 was identified by immunoprecipitation with anti-SNAP-23 antibodies and autoradiography. Weak but consistent phosphorylation of SNAP-23 was detected in non-stimulated platelets (Fig. 2). Thrombin activation but not

RESULTS

SNAP-23/25/29 Family Proteins in Human Platelets—The expression of SNAP-25 family proteins was examined by immunoblotting (Fig. 1A). SNAP-23 was readily detected in platelet samples containing as little as 1.5 μg of protein, but only a very faint band was seen in the brain lysate samples containing 20-fold more protein. In contrast, SNAP-25 (which migrated faster than SNAP-23) was expressed robustly in the brain and only trace amounts were detected in the platelet samples. SNAP-29 was detected as a single band in the brain sample with a relative mass of ~33 kDa. Two major bands of slightly larger relative mass, 34–35 kDa, were seen in the platelet that were not detected when the primary antibody was omitted (data not shown). Platelet and brain SNAP-29 proteins may differ because of alternate transcript splicing or post-translational modification. These data indicate that SNAP-23 is a major SNAP-23/25/29 family protein in the platelet.

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**Fig. 1.** Expression of SNAP-23/25/29 family proteins in human platelets. A, immunoblots of human platelet (plt) and brain lysates probed with antibodies against SNAP-23 (rabbit polyclonal antibodies against human SNAP-23), SNAP-29 (rabbit polyclonal antibodies against rat SNAP-29; Synaptic Systems, 111302), and SNAP-25 (monoclonal antibodies against human SNAP-25) are shown. The amounts of platelet and brain proteins loaded on the gel are indicated. The positions of pre-stained molecular mass markers are shown. B, distribution of SNAP-23 relative to other proteins in platelet subcellular fractions. Non-stimulated platelets were disrupted by nitrogen cavitation, and subcellular fractionation was performed by sucrose gradient ultracentrifugation. Immunoblots were probed with antibodies against the indicated proteins. Positions of pre-stained molecular mass markers are indicated on the right. The bottom row shows the distribution of [\(^{14}\text{C}\)]-serotonin in the subcellular fractions as the percent of the total [\(^{14}\text{C}\)]-serotonin content. C, effect of SNAP-23 antibodies on granule exocytosis in permeabilized platelets. Streptolysin O-permeabilized platelets were incubated with anti-SNAP-23 antibodies or pre-immune rabbit IgG, and granule secretion was induced by increasing the free calcium-ion concentration to 10 μM. Granule secretion was monitored by measuring P-selectin expression with phycoerythrin-conjugated anti-CD62 antibodies and flow cytometry. Dense granule secretion was measured in platelets loaded with [\(^{14}\text{C}\)]-serotonin by scintillation counting. Hexosaminidase activity was measured in the platelet supernatants to assess lysosomal secretion. The data indicate mean ± S.D. percentage secretion relative to control, pre-immune IgG (n = 3, independent experiments with platelets from different donors).

**Fig. 2.** SNAP-23 is phosphorylated in thrombin-activated human platelets. Platelets were incubated with \(^{32}\text{P}\)-inorganic phosphate to label the platelet ATP pool. The platelets were activated with 1 unit/ml thrombin (1 min) or passivated with 1 μM PGI\(_2\) (1 min) or left untreated. After platelet lysis, platelet proteins were immunoprecipitated with anti-SNAP-23 or irrelevant (control) antibodies, separated by SDS-PAGE, and transferred to PVDF membranes. The membranes were analyzed by phosphorimaging (upper panel) or by immunoblotting with anti-SNAP-23 antibodies and chemifluorescence imaging (same membrane, lower panel). IP, immunoprecipitation; AB, antibody.

PGI\(_2\) passivation significantly increased the phosphorylation of SNAP-23 (Fig. 2).

**Kinetics of Granule Secretion and SNAP-23 Phosphorylation in Activated Platelets**—If phosphorylation of SNAP-23 plays a role in platelet exocytosis, it must precede or occur synchronously with degranulation. To examine phosphorylation and granule secretion in tandem, the kinetics of platelet activation were slowed by preventing aggregation (non-stirred samples and EDTA) and by performing the assays at room temperature (22 ± 1.5°C). The time-dependent secretion from dense (Fig. 3A) and α (Fig. 3B) granules was examined with various doses of thrombin (0.03–1 unit/ml) in the same platelet sample. Secretion from α granules progressively increased during the 10 min of observation. In contrast, dense granule secretion reached a plateau between 0.5 and 1 min even if maximum secretion was not reached. This is an interesting, and, to our knowledge, not yet reported, difference in the kinetics of platelet α and dense granule secretion. The kinetics of α and dense granule secretion and SNAP-23 phosphorylation were examined in platelets stimulated with saturating (1 unit/ml) and non-saturating (0.03 unit/ml) thrombin concentrations (Fig. 3, C and D). SNAP-23 phosphorylation showed similar kinetics to dense granule secretion (Fig. 3, C and D). Both dense granule secretion and the phosphorylation of SNAP-23 reached a maximum shortly after thrombin activation and remained flat afterward. These data showed that SNAP-23 phosphorylation was an early event in the activated platelet. The parallel between SNAP-23 phosphorylation and granule secretion suggested that phosphorylation of SNAP-23 may play a role in secretion.

**SNAP-23 Phosphorylation and Kinase Inhibition**—SNAP-23 has several potential phosphorylation sites; however, immunoblotting with anti-phosphotyrosine antibodies (4G10; Upstate Biotechnology, Inc.) did not reveal phosphotyrosine in SNAP-23 immunoprecipitated from thrombin-activated platelets (data not shown). Phosphoamino acid analysis of platelet SNAP-23 showed that Ser (>80%) and to a lesser extent Thr (15 ± 4%, average ± S.D., n = 4) residues were phosphorylated in activated platelets (Fig. 4). A similar pattern of Ser versus Thr phosphorylation was found in PGI\(_2\)-passivated (1 μM, 1 min) platelets (data not shown). Kinase inhibitors were used to gather further information about which kinases may or may not be involved in the phosphorylation of SNAP-23. Table I shows that inhibitors of various serine/threonine kinases had no effect on the phosphorylation of platelet SNAP-23. However, pre-incubation of platelets with PKC inhibitors or with the PKC activator phorbol 12-myristate 13-acetate (PMA) inhibited or further stimulated the phosphorylation of SNAP-23, respectively, providing evidence that SNAP-23 phosphorylation was PKC-dependent.
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Fig. 3. Kinetics of granule secretion and SNAP-23 phosphorylation in platelets activated with various concentrations of thrombin. A and B, dense (A) and α (B) granule secretion was measured in the same platelet samples preloaded with [14C]serotonin. Secretion was induced with 0.03–1 unit/ml thrombin (as shown) for the time indicated in non-stirred samples with 1 mM EDTA (to prevent platelet aggregation). Secretion from α granules was monitored by measuring P-selectin expression with phycoerythrin-conjugated anti-CD62 antibodies and flow cytometry. The secretion was induced by 1 unit/ml in 10 min defatted as total secretion, 100%. The [14C]serotonin secreted by platelets activated with 1 unit/ml for 10 min represented 80–90% of the [14C]serotonin content of platelet samples lysed in 0.4% Triton X-100. The means ± S.D. are shown. Data are representative of three independent experiments.

C and D, kinetics of α (□) and dense (□) granule secretion and SNAP-23 phosphorylation (∗) induced by 1 unit/ml (C) and 0.03 unit/ml (D) thrombin. Secretion was measured as above. SNAP-23 phosphorylation (∗) was analyzed by immunoprecipitation of SNAP-23 from platelets with 32P-labeled ATP pool, SDS-PAGE, electroblotting, and phosphorimaging. The results are expressed as -fold increase relative to phospho-SNAP-23 in non-stimulated platelets. Data (means ± S.D.) are representative of two independent experiments.

Fig. 4. SNAP-23 is phosphorylated on Ser/Thr residues in activated platelets. A, model autoradiogram of a two-dimensional phosphoamino acid separation of SNAP-23 isolated from platelets activated with thrombin (1 unit/ml, 1 min). The x shows the origin, where hydrolyzed platelet SNAP-23 and non-radioactive phosphoamino acid standards (Ser(P), Thr(P), Tyr(P), 1 μg each) were loaded on the thin layer chromatography plate. Dashed circle shows the position of Tyr(P), identified by ninhydrin staining. The positions of Ser(P) and Thr(P) identified by ninhydrin staining (not shown in the figure) overlap with the Ser(P) and Thr(P) spots in the autoradiogram. Radioactive spots above the origin represent partially hydrolyzed protein fragments. This autoradiogram is representative of four independent experiments. P-Ser, Ser(P); P-Thr, Thr(P); P-Tyr, Tyr(P).

Recombinant SNAP-23 was phosphorylated by various recombinant PKC isozymes (α, βI, βII, γ, δ, ε, η, ζ). PKC isozymes α, γ, and δ showed the most robust phosphorylation of r-SNAP-23. Approximately 5-fold less phosphorylation was seen with βI, βII, and ε isozymes, and negligible phosphorylation was detected with the ζ isozyme (data not shown). The stoichiometry of phosphate incorporation by purified PKC (mixture of α, β, and γ isozymes) ranged from 0.07 to 0.3 mol of P/mol of SNAP-23, which may reflect different ratios of properly folded versus misfolded r-SNAP-23 in different samples of bacterially expressed proteins. Nevertheless, the incorporation of up to 0.3 mol of P/mol of recombinant protein suggested that SNAP-23 was a substrate of PKC. The results of phosphoamino acid analysis of PKC-phosphorylated r-SNAP-23 were similar to the results obtained with platelet SNAP-23 because phospho-Ser was the most abundant residue followed by smaller amounts of phospho-Thr (Fig. 4 and data not shown).

To further analyze whether PKC may phosphorylate SNAP-23 in platelets, we compared the phosphopeptide fingerprints (two-dimensional analysis of tryptic digests) of SNAP-23 immunoprecipitated from thrombin-activated platelets and r-SNAP-23 phosphorylated with purified PKC. If PKC phosphorylated SNAP-23 in the platelet, the phosphopeptide fingerprints from platelet SNAP-23 and r-SNAP-23 should overlap. Trypsin digestion of PKC-phosphorylated r-SNAP-23 resulted in three major phosphopeptides (Fig. 5). Interestingly, PKC-phosphorylation of SNAP-23, which was engaged in a complex with syntaxin 4 and VAMP 2, resulted in only one major phosphopeptide. This showed that PKC differentially phosphorylated monomeric SNAP-23 and SNAP-23 when it was engaged in SNARE complexes. In the tryptic digests of SNAP-23 immunoprecipitated from activated platelets, there were five major phosphopeptides (Fig. 5). Three of these phosphopeptides (2, 3, and 4 in Fig. 5) co-migrated with the phosphopeptides in the fingerprints of r-SNAP-23 phosphorylated with PKC. These results supported the hypothesis that PKC is one of the kinases that may phosphorylate SNAP-23 in the platelet.

Identification of Phosphorylated Residues in Platelet SNAP-23 and PKC-phosphorylated Recombinant SNAP-23—To identify phosphorylation sites, SNAP-23 was afffinity-purified from thrombin-activated or PGL-p-passivated platelets. After separation by SDS-PAGE, SNAP-23 was excised from the gel and analyzed by MS/MS. In SNAP-23 from activated platelets, Ser161 was unambiguously identified as a phosphorylation site in two singly phosphorylated peptides (EDEMEENLTQVGSILGNLKI and TNDAREDEEMNLTQVGSILGNLKI). Two other singly phosphorylated peptide sequences were detected, each revealing the sequence AHQITDESLES23T24RR. Determination of the precise phosphorylation site among the two residues (Ser23/Thr24) can be ambiguous, although it is definitive that at least one of them was phosphorylated. Both the doubly and triply charged precursor ions were subjected to MS/MS. The triply charged MS/MS spectrum indicated a slight preference for phosphorylation of Ser23 over Thr24; however, phosphorylation of Thr24 could not be ruled out. Ser23/Thr24 and Ser161 (Fig. 6, bold and underlined) are each located on one of the four α-helices in the ternary SNARE complex produces 16 highly conserved layers of interacting amino acid side chains that are numbered in Fig. 6 as previously described (3, 4). Ser23 is just N-terminal to layer −7, Thr24 is in layer −7, and Ser161 is between layers −6 and −5.

No phosphorylation was detected in SNAP-23 from PGL-p-passivated platelets despite the very high peptide coverage of SNAP-23 (>88%; in these types of experiments, 40–60% coverage is typical). This was probably because of the low phosphate stoichiometry.

The phosphorylation sites in PKC-phosphorylated r-SNAP-23 were also identified. Similar to platelet SNAP-23, a singly
The effect of kinase inhibitors or activators on thrombin-induced SNAP-23 phosphorylation in human platelets

Table I

| Thrombin | Inhibitor/activator | SNAP-23 phosphorylation (means ± S.D) |
|----------|---------------------|--------------------------------------|
| +        | None                | 100 ± 4                              |
| –        | None                | 17 ± 6                               |
| +        | Ro 31-8220, 1 μM (PKC inhibitor) | 34 ± 2                              |
| +        | PMA, 1 μM (PKC activator) | 172 ± 13                             |
| +        | LY294, 20 μM (phosphoinositide 3-kinase inhibitor) | 102 ± 7                             |
| +        | PD169316, 1 μM (p38 MAPK inhibitor) | 114 ± 4                             |
| +        | KN92, 10 μM (calcium/calmodulin kinase II inhibitor) | 128 ± 13                             |
| +        | PD98059, 20 μM (MAPK kinase inhibitor) | 115 ± 7                             |
| +        | Odemouine, 50 μM (p44 MAPK inhibitor) | 121 ± 14                             |
| +        | None (control, 0.1% Me2SO) | 119 ± 15                             |

Platelets with a 32P-labeled ATP pool were incubated with inhibitors for 30 min, or with PMA for 1 min, before activation with 1 unit/ml thrombin for 1 min. After platelet lysis, platelet proteins were immunoprecipitated with anti-SNAP-23 or pre-immune IgG, separated by SDS-PAGE, transferred to PVDF membranes. Membranes were analyzed by phosphorimaging and by immunoblotting (anti-SNAP-23 antibodies and chemiluminescence imaging). To compare equal amounts of SNAP-23 protein, radioactivity pixel numbers were normalized to the amounts of immunoreactive SNAP-23 protein. The results were expressed as percentage of phosphorylation. Thrombin-stimulated samples with no inhibitor were defined as 100%. All compounds, except Ro 31–8220, were dissolved in Me2SO, up to 0.1% final concentration in the assay.

Fig. 5. Comparison of the phosphopeptide fingerprints of SNAP-23 phosphorylated in thrombin-activated human platelets as well as PKC-phosphorylated r-SNAP-23. PKC and [γ-32P]ATP were used to phosphorylate r-SNAP-23 and ternary complexes of recombinant SNAP-23, syntaxin 4, and VAMP 2. Platelets were incubated with 32P-labeled inorganic phosphate to label the platelet ATP pool. Platelets were activated with thrombin, and SNAP-23 was immunoprecipitated from the platelet lysate. Recombinant and platelet-derived SNAP-23 were separated from other proteins in the samples by SDS-PAGE and electroblotted to PVDF membranes. The immobilized SNAP-23 was digested with trypsin and the peptides were separated on TLC plates by two-dimensional electrophoresis and chromatography. Horizontal, electrophoresis at pH 1.9, separation by charge/mass. Vertical, chromatography, separation by hydrophobicity. 32P-Phosphopeptides were detected by autoradiography. Upper row shows representative fingerprints of three independent experiments. Lower row shows the consensus pattern of major radioactive phosphopeptide spots in the fingerprints. Co-migrating phosphopeptides are indicated by the same number in different samples.

Phosphorylated peptide, EDEMEENLTQGSLGNLK, unambiguously identified Ser161 as a phosphorylation site whereas two other singly phosphorylated peptide sequences showed the sequence AHQITIDLESSTR, identifying Ser23 and/or Thr24 as phosphorylation sites. In addition, two singly phosphorylated peptides (TKNFESGKAYK and NFESGKAYK) identified a third phosphorylation site, Ser46, in PKC-phosphorylated r-SNAP-23. Ser95 is located in a region that connects the two SNAP-23 motifs in SNAP-23. Ser23/Thr24 and Ser95 are located in the two consensus PK phosphorylation sites in SNAP-23.

The fact that both of the phosphorylation sites identified in platelet SNAP-23 were also found to be phosphorylation sites in PKC-phosphorylated r-SNAP-23 further supported the hypothesis that PKC phosphorylated SNAP-23 in the activated platelet.

Mutation of SNAP-23 Phosphorylation Sites Alters Syntaxin 4 Binding—Ser or Thr residues are commonly mutated to Asp to examine how phosphorylation of a protein may affect function (40–42). We determined whether phosphorylation may alter SNARE protein interactions by examining the binding of SNAP-23 pseudo-phosphorylation mutants (Ser23 → Asp and Ser161 → Asp) to syntaxin 4. When compared with native SNAP-23, two different independent clones of the SNAP-23 (Ser23 → Asp/Thr24 → Asp) mutant showed significantly reduced binding (up to 50%) to syntaxin 4 at all times, concentrations (10, 20, and 40 μg/ml), and temperatures (4 and 22 °C) tested (Fig. 7; data from 10 and 20 μg/ml concentrations and 4 °C not shown). In contrast, two different clones of the SNAP-23 (Ser161 → Asp) mutant showed minor differences when compared with native SNAP-23 under the same conditions (Fig. 7, and data not shown). This provides evidence that phosphorylation at the Ser23/Thr24 sites may alter interactions that are important for SNARE complex formation. Because we have shown that soluble forms of VAMPs inhibit Ca2+-triggered secretion in permeabilized platelets (24), we hypothesized that soluble native SNAP-23 would also inhibit platelet secretion, whereas the SNAP-23 (Ser23 → Asp/Thr24 → Asp) mutant would not, because of impaired SNARE complex interactions. However, these experiments were inconclusive because, as previously reported (21, 22), recombinant full-length SNAP-23 had no detectable effects on secretion (nor did the SNAP-23 mutants). We attempted to gather further evidence for the functional effects of SNAP-23 phosphorylation in permeabilized platelets by using SNAP-23 peptides as decoy substrates, similar to the approach used to examine the role of myristoylated alanine-rich C kinase substrate in platelet secretion (43). Six peptides were produced that represented the native N- and C-terminal SNARE motifs of SNAP-23 as well as the Asp or Ala mutants in positions Ser23/Thr24 and Ser161. Unfortunately, these experiments were also indeterminate because none of these peptides, including the native SNAP-23 peptides, had any effect on granule secretion in permeabilized platelets.

Discussion

These studies establish SNAP-23 as a major SNAP-23/25/29 family protein in human platelets. Specific anti-SNAP-23 antibodies inhibited secretion from all three types of platelet granules in permeabilized cells, underlining the important role SNAP-23 plays in the mechanism of platelet granule secretion. This finding was in agreement with earlier reports (21, 22, 39). Although it has been suggested that platelets contained a significant pool of soluble, non-membrane-associated SNAP-23 (22), our studies indicate that SNAP-23 was a membrane-
Phosphorylation of SNAP-23 in Activated Human Platelets

PKC may regulate platelet secretion through phosphorylation of SNAREs, including SNAP-23 and syntaxin 4 (28), as well as through phosphorylation of SNARE regulators, including FSP/Munc18c (18).

Our finding that SNAP-23 phosphorylation kinetics paralleled or preceded the rate of granule release suggested that SNAP-23 phosphorylation may play a role in platelet granule secretion. We have shown that platelet activation by thrombin or PMA decreased the binding of SNAP-23 with syntaxin 4 (28). Additionally, phosphatase inhibitors further decreased syntaxin 4-SNAP-23 binding induced by cell activation, whereas PKC inhibitors restored syntaxin 4-SNAP-23 binding to that seen in non-stimulated platelets (28). In this interaction syntaxin 4-SNAP-23 binding may be mediated by phosphorylation of SNAP-23 or by phosphorylation of syntaxin 4 or both. It is not yet clear how decreased SNAP-23-syntaxin 4 binding relates to membrane fusion events required for platelet secretion. We (28) and others (20) have found decreased amount of SNARE complexes in activated versus non-stimulated platelets, but the relative amount of cis- and trans-SNARE complexes are unknown. Phosphorylation of monomeric SNAREs versus SNAREs in ternary complexes is also likely to be different (Fig. 5), because the formation of the ternary SNARE complex is accompanied by dramatic conformational changes (4, 46, 47). For example, a novel serine/threonine kinase, SNAK, phosphorylates only SNAP-23 that is not assembled into SNARE complexes (48). Complexed and non-complexed SNAREs may be phosphorylated by different kinases, by different kinetics, at different time points in the course of regulated exocytosis. This further complicates studies of SNARE phosphorylation and its function in in vivo situations where monomeric SNAREs and various cis- and trans-SNARE complexes as well as SNAP-protein-SNARE regulator complexes co-exist.

Still, identification of SNARE protein residues, which are phosphorylated or dephosphorylated in the course of intracellular membrane trafficking/fusion events in secretory cells in vivo, is integral to fully understanding the molecular mechanisms of regulated exocytosis. Studies of SNAP-25 have shown that this family of proteins contributes two coiled-coil domains to the four-helix bundle of the ternary SNARE complex (4, 46). Ser\(^{23}/\text{Thr}^{24}\) and Ser\(^{161}\) reside on the first (N-terminal) and the second coiled-coil domains of SNAP-25, respectively. The association of the four \(\alpha\)-helices in the ternary SNARE complexes produces 16 highly conserved layers of interacting amino acid side chains (Fig. 6; layer numbers as in Refs. 3 and 4). Residues corresponding to Ser\(^{23}\) and Thr\(^{24}\) in SNAP-23 are highly conserved, and the side chains of these residues face inward in the ternary complex (very small relative surface accessibility) (3). As such it is likely that Ser\(^{23}/\text{Thr}^{24}\) can be phosphorylated in monomeric SNAP-23 but not when SNAP-23 is engaged in the ternary complex. Consistent with this we found more major phosphopeptide spots in the fingerprints of PKC-phosphorylated r-SNAP-23 monomers than in PKC-phosphorylated r-SNAP-23, which was engaged in a complex with syntaxin 4.

A consistent, low level phosphorylation of SNAP-23 was detected in non-activated platelets. This weak SNAP-23 phosphorylation probably is characteristic of resting platelets, rather than a result of unintended partial activation of the platelet samples, because the phospho-SNAP-23 signals were the same in non-treated and PGL\(_2\)-treated platelets. Platelet activation with thrombin resulted in a significant increase in the phosphorylation of SNAP-23. SNAP-23 phosphorylation was an early event after activation of the platelet. Indeed, the kinetics of SNAP-23 phosphorylation paralleled the kinetics of granule secretion, supporting the hypothesis that SNAP-23 phosphorylation may play a role in platelet granule exocytosis.

To identify the kinase(s) that may be involved in the phosphorylation of SNAP-23 in platelets, we examined the effects of various inhibitors and activators. Phosphorylation of SNAP-23 in thrombin-activated platelets was inhibited by PKC inhibitors but was not affected by inhibitors of phosphoinositide 3-kinase, p38 and p44 MAPK, MAPK kinase, or calcium/calmodulin kinase II. In addition, the PKC activator PMA stimulated phosphorylation of SNAP-23. Various PKC isoforms phosphorylated r-SNAP-23, and phosphopeptides derived from platelet SNAP-23 (immunoprecipitated from activated platelets) co-migrated with phosphopeptides derived from PKC-phosphorylated r-SNAP-23. Both of the two phosphorylation sites identified on SNAP-23 from activated platelets, Ser\(^{23}\)/Thr\(^{24}\) and Ser\(^{161}\), were phosphorylated by PKC in r-SNAP-23. Indeed, Ser\(^{23}/\text{Thr}^{24}\) is part of a consensus PKC phosphorylation site. Taken together these data strongly suggest that PKC phosphorylates SNAP-23 in the thrombin-activated platelet. A recent report provided direct evidence that PKC \(\alpha\) isoform was essential for both platelet \(\alpha\) and dense granule secretion (45).

A chart shows the cpm values over different time points (15 min, 30 min, 75 min) for samples from different conditions: D161, D162, D234, D244, D161/2, D162/3, D234/4, D161/234/4, and m/ave. The data are representative of two independent experiments with means \(\pm\) S.D. shown.

**Fig. 6. Platelet SNAP-23 phosphorylation sites.** Partial sequence alignment of SNAP-23 and SNAP-25. Alignment is restricted to the region encompassing the interacting layers of the fusion complex. (Crystallographic data have been published for the neuronal SNARE complex, ternary complex of SNAP-25, syntaxin 1, and VAMP 2.) The layers are indicated by arrows and numbered as in Refs. 3 and 4. The phosphorylation sites, Ser\(^{23}/\text{Thr}^{24}\) (Ser\(^{23}\) or Thr\(^{24}\)) and Ser\(^{161}\), identified in SNAP-23 by mass spectrometric analysis are indicated by underlined, boldface type.

**Fig. 7. Binding of syntaxin 4 to mutants that mimic SNAP-23 phosphorylation.** Native and mutated SNAP-23 were incubated in microtiter plates coated with syntaxin 4. The binding of SNAP-23 (40 \(\mu\)g/ml, 22 °C, indicated times) was determined with anti-SNAP-23 antibodies as detected by 125I-protein A. Two different clones (1# and 2#) with the correct sequences for the SNAP-23 mutants (Ser\(^{23}\) \(\rightarrow\) Asp/Thr\(^{24}\) \(\rightarrow\) Asp and Ser\(^{161}\) \(\rightarrow\) Asp) were compared with native SNAP-23. Background binding was less than 200 cpm as measured in wells without coated syntaxin 4 and SNAP-23. The data are representative of two independent experiments with means \(\pm\) S.D. shown.
and VAMP 2 (Fig. 5). Phosphorylation of SNAP-23 on Ser\(^{23}\)/Thr\(^{24}\) may alter SNARE complex formation because, during assembly of the four-helix bundle, the 16 interacting layers may form sequentially in a zipper-like fashion, beginning at the N terminus (layer −7) and progressing toward the C terminus (49). Consistent with this hypothesis, we found that a SNAP-23 mutant (Ser\(^{23}\) → Asp/Thr\(^{24}\) → Asp), designed to mimic phosphorylation at the Ser\(^{23}\)/Thr\(^{24}\) sites, reduced syntaxin 4 binding. In contrast, residues in Q-SNAREs that correspond to Ser\(^{161}\) in SNAP-23 show high sequence variability and high surface accessibility in the ternary complex (3). This is in line with our finding that Asp\(^{161}\) mutants of SNAP-23 did not show significant reduction in their binding to syntaxin 4. It would be predicted that Ser\(^{161}\) can be phosphorylated in both SNAP-23 monomers and in SNAP-23 engaged in a ternary complex; however, a phosphoseryl on the surface of the ternary SNARE complex between layers −5 and −6 would be a specific characteristic of complexes with SNAP-23. Further experiments will be required to determine whether Ser\(^{161}\) phosphorylation affects the interaction of the SNAP-23 complex with regulatory proteins.

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