Phosphorylation of SNAP-23 Regulates Exocytosis from Mast Cells*

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Regulated exocytosis is a process in which a physiological trigger initiates the translocation, docking, and fusion of secretory granules with the plasma membrane. A class of proteins termed SNAREs (including SNAP-23, syntaxins, and VAMPs) are known regulators of secretory granule/plasma membrane fusion events. We have investigated the molecular mechanisms of regulated exocytosis in mast cells and find that SNAP-23 is phosphorylated when rat basophilic leukemia mast cells are triggered to degranulate. The kinetics of SNAP-23 phosphorylation mirror the kinetics of exocytosis. We have identified amino acid residues Ser95 and Ser120 as the major phosphorylation sites in SNAP-23 in rodent mast cells. Quantitative analysis revealed that ~10% of SNAP-23 was phosphorylated when mast cell degranulation was induced. These same residues were phosphorylated when mouse platelet degranulation was induced with thrombin, demonstrating that phosphorylation of SNAP-23 Ser95 and Ser120 is not restricted to mast cells. Although triggering exocytosis did not alter the absolute amount of SNAP-23 bound to SNAREs, after stimulation essentially all of the SNAP-23 bound to the plasma membrane SNARE syntaxin 4 and the vesicle SNARE VAMP-2 was phosphorylated. Regulated exocytosis studies revealed that overexpression of SNAP-23 phosphorylation mutants inhibited exocytosis from rat basophilic leukemia mast cells, demonstrating that phosphorylation of SNAP-23 on Ser120 and Ser95 modulates regulated exocytosis by mast cells.

The RBL-2H31 mast cell line has been extensively studied as a model not only for mast cell biology but also as a paradigm for regulated exocytosis from non-neuronal cells (2). Stimulation of the high affinity IgE receptor, FceRI, on these cells by cross-linking initiates a signal transduction cascade that culminates in secretory granule fusion with the plasma membrane, thereby liberating a variety of inflammatory mediators (3, 4). Numerous proteins necessary for the tethering, docking, and fusion steps between various membrane compartments in eukaryotic cells have been described. Among those, SNAREs (soluble NSF-attachment protein receptors) are a large family of membrane-associated proteins essential for membrane-membrane fusion (5, 6). These proteins include members of the vesicle-associated synaptobrevin/VAMP family as well as members of the syntaxin and SNAP-23 families of “target” membrane SNAREs. The current model proposes that while vesicles are docked on the target membrane, SNAREs from the donor or vesicle membrane (v-SNAREs) form trans-SNARE complexes with their cognate SNARE partners on the opposing target membrane. Structurally, the exocytic SNARE complex is a trimolecular protein complex containing one member of the VAMP, syntaxin, and SNAP-23 family, each contributing to the formation of a four-helix coiled-coil bundle (7, 8) whose formation is sufficient for in vitro membrane fusion (9).

Given that SNAREs play a central role in the membrane fusion process, it is likely that their function is modulated in vivo. In particular, protein kinases, which have been extensively associated with the regulation of exocytosis (10), could participate in SNARE function by phosphorylating residues essential in SNARE complex assembly or the binding of SNARE regulatory proteins (6, 11, 12). Members of the syntaxin (13–15) and SNAP-23/25 (14, 16, 17) family of proteins are substrates for various protein kinases in vitro, although precise SNARE substrates in vivo and the physiological consequences of SNARE phosphorylation are not clear.

The plasma membrane localized target SNAREs, SNAP-23 and syntaxin 4, have been shown to be important mediators of granule/plasma membrane fusion in mast cells (18–21) and platelets (22, 23). Pombo et al. (15) found that syntaxin 4 was constitutively phosphorylated in RBL mast cells and the extent of syntaxin 4 phosphorylation was not altered during secretion. By contrast, using human platelets Chung et al. (24) found that thrombin stimulation resulted in phosphorylation of this same SNARE protein. In neither of these studies were the syntaxin 4 phosphorylation sites identified or the physiological impor-

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1 The abbreviations used are: RBL, rat basophilic leukemia; SNARE, SNAP receptor; SNAP, soluble NSF attachment protein; NSF, NEM-sensitive fusion protein; PRC, protein kinase C; DNP, dinitrophenol; GST, glutathione S-transferase; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; VAMP, vesicle-associated membrane protein.
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EXPERIMENTAL PROCEDURES

Antigens and Cell Culture—Rabbit antisera recognizing the SNAP-23 carboxyl terminus have been described (25). A polyclonal rabbit antisera recognizing rat syntaxin 3 (26) was the generous gift of Dr. E. John Simons (Johns Hopkins University, Baltimore, MD). The polyclonal rabbit antisera recognizing rat syntaxin 4 was generated by immunizing rabbits with GST fusion protein of rat syntaxin 4 (amino acids 1–274). This antiserum showed no detectable cross-reactivity with rat syntaxin 2 or rat syntaxin 3 and was used in all immunoprecipitation and immunoblotting studies. Antiserum recognizing the phospho-SNAP-23-Ser250 was generated by immunizing rabbits with the synthetic peptide Val-Ser-Lys-Gln-Pro-phospho-Ser-Arg-Ile-Thr (corresponding to amino acids 115–126 of rat SNAP-23). Antisera recognizing the phospho-SNAP-23-Thr250 was generated by immunizing rabbits with the synthetic peptide Thr-Lys-Asn-Phe-Glu-phospho-Ser-Gly-Lys (corresponding to amino acids 90–102 of rat SNAP-23). The VAMP-2 monoclonal antibody cl 69.1 was obtained from Synaptic Systems (Goettingen, Germany). Unless indicated, anti-DNP-IgE was from Sigma and PhosphoImager analysis analysis.

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METABOLIC LABELING OF CELLS—Rat mast cells were purified from isopropanol 1-thio-beta-galactopyranoside-in- duced B1.21.E4.1 cells using standard protocols. Briefly, GST and GST-SNAP-23 were isolated from 200-μl bacteria lysates with 10 μl (1:1) of glutathione-Sepharose beads (Amersham Biosciences). GST proteins were eluted with 30 μl of 1 M reduced glutathione and phosphorylated for 30 min at 30 °C with 25 ng of purified PKC (Promega) in a reaction buffer of 20 mM Heps (pH 7.4), 1.67 mM CaCl2, 1 mM phosphoethanolamine (Phosphoethanolamines, Sigma) for 5 h in 1 ml of phosphate-free DMEM. Cells were then stimulated for 5 min with DNP-BSA as described above. Cells were washed with ice-cold Hanks’s balanced salt solution and tissue culture plate-bound cells were frozen until further use.

In Vitro Phosphorylation Reactions—GST and GST-SNAP-23 proteins were purified from isopropanol 1-thio-beta-galactopyranoside-induced B1.21.E4.1 cells using standard protocols. Briefly, GST and GST-SNAP-23 were isolated from 200-μl bacteria lysates with 10 μl (1:1) of glutathione-Sepharose beads (Amersham Biosciences). GST proteins were eluted with 30 μl of 1 M reduced glutathione and phosphorylated for 30 min at 30 °C with 25 ng of purified PKC (Promega) in a reaction buffer of 20 mM Heps (pH 7.4), 1.67 mM CaCl2, 1 mM phosphoethanolamine (Phosphoethanolamines, Sigma) for 5 h in 1 ml of phosphate-free DMEM. Cells were then stimulated for 5 min with DNP-BSA as described above. Cells were washed with ice-cold Hanks’s balanced salt solution and tissue culture plate-bound cells were frozen until further use.

Phosphorylation of SNAP-23 phosphorylates regulated exocytosis in mast cells. We find that phosphorylation of SNAP-23 inhibits the extent of regulated exocytosis from RBL mast cells. We also find that phosphorylated SNAP-23 preferentially associated with syntaxin 4 and VAMP-2 after exocytosis was triggered. These data suggest that phosphorylation of SNAP-23 is an important post-translational modification of a critical SNARE that regulates mast cell exocytosis.

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Subcellular Fractionation—RBL cells were harvested by trypsination and washed in phosphate-buffered saline. The cells were re-suspended in hypotonic buffer (10 mM Tris, 10 mM KC1, 1 mM EDTA, 0.5 mM MgCl2, pH 7.4) and were disrupted by repeated passage of cells through a 25-gauge syringe. Nuclei and unbroken cells were removed by centrifugation at 1000 × g and the post-nuclear supernatant was subjected to centrifugation at 100,000 × g for 1 h at 4 °C to isolate membranes (pellet) and cytosol (supernatant). In some experiments RBL cells were transfected with pEGFP (Clontech) to allow expression of the cytosolic marker protein GFP. The membrane pellet and cytosolic supernatant were brought to the same volume in hypotonic buffer and each was adjusted to a final concentration of 1% Triton X-100. Equal portions of each fraction were analyzed by SDS-PAGE and immunoblotting.

Mast Cell Exocytosis Assay—The degranulation in RBL-2H3 cells was monitored by measuring the β-hexosaminidase activity released from cells grown in 6- or 12-well plates. IgE-sensitized cells were washed twice with RPMI and mock-stimulated or stimulated by the addition of DNP-BSA. The supernatant was collected and the cells were lysed in phenol red-free RPMI containing 0.2% Triton X-100 to determine the total enzyme content. A colorimetric assay with p-nitrophenyl-N-acetyl-β-D-galactosaminide (Sigma) as the substrate was used to measure the amount of β-hexosaminidase released into the medium and remaining in the cells as described previously (20). The β-hexosaminidase activity released was expressed as a percentage of the activity released into the medium relative to the total activity (released plus cell-associated).

In some experiments, RBL cells were transfected by electroporation with an expression vector of human growth hormone (2 μg) together with empty pcMV vector or pcMV-FLAG-SNAP-23 (20 μg). After overnight culture, the cells were sensitized with IgE and mock-stimulated or stimulated with DNP-BSA as described previously (20). The growth hormone released into the medium or remaining cell associated was determined using a human growth hormone enzyme-linked immunosorbent assay (Roche Diagnostics Corp.) as described previously (29). For quantitative experiments, statistical analyses were carried out by using a Student’s t test. Results were considered significant when a p value of less than 0.05 was obtained.

Platelet Preparation and Measurement of Thrombin-induced Secretion—Mouse platelets were isolated as described previously (30). Briefly, blood was obtained from the right ventricle of the heart of sacrificed C57BL/6 mice. The blood was mixed with 1.8% sodium citrate (pH 7.4) to a final concentration of 0.18%. Platelet-rich plasma was centrifuged at 100,000 × g for 1 h at 4 °C to isolate membranes (pellet) and cytosol (supernatant). In some experiments RBL cells were transfected with pEGFP (Clontech) to allow expression of the cytosolic marker protein GFP. The membrane pellet and cytosolic supernatant were brought to the same volume in hypotonic buffer and each was adjusted to a final concentration of 1% Triton X-100. Equal portions of each fraction were analyzed by SDS-PAGE and immunoblotting.

Platelets were assayed for dense core release of [3H]β-hydroxytryptamine, lysosomal release of β-hexosaminidase, and α-granule release of platelet factor IV, as described previously (31). Platelets were activated by the addition of thrombin (0.5 units/ml; Chronolog) in a Hepes/Tyrode’s release buffer containing 0.7 mM CaCl2 at 25 °C for the indicated times. The data were tabulated as the percent release compared with the total present in each reaction. The background release of each marker in unstimulated platelets was subtracted from that obtained after thrombin stimulation to yield net granule marker release. A parallel set of reactions was solubilized in SDS-PAGE sample buffer for analysis by immunoblotting.

RESULTS

SNAP-23 Is Phosphorylated in Stimulated RBL Cells—RBL cell exocytosis can be triggered by cross-linking of surface FcεRI receptors for IgE by the appropriate antigen (3, 4). To determine whether SNAREs were phosphorylated during physiological stimulation in mast cells, RBL-2H3 cells were metabolically labeled with [32P]orthophosphate prior to cross-linking surface FcεRI receptors for IgE by the appropriate antigen (3, 4). After over-night culture, the cells were sensitized with IgE and mock-stimulated or stimulated with DNP-BSA as described previously (20). The growth hormone released into the medium or remaining cell associated was determined using a human growth hormone enzyme-linked immunosorbent assay (Roche Diagnostics Corp.) as described previously (29). For quantitative experiments, statistical analyses were carried out by using a Student’s t test. Results were considered significant when a p value of less than 0.05 was obtained.

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Quantitative immunoblot analysis of the SNAP-23 immunoprecipitate showed no changes in the total amount of SNAP-23 after stimulation of RBL cells, however, the SNAP-23 band became heterogeneous and higher molecular weight isoforms of SNAP-23 appeared after stimulation. (The more rapidly migrating band in the SNAP-23 immunoblot is an amino-terminal degradation product of SNAP-23 that is only seen in rat tissues. B. RBL cells were coated with DNP-specific IgE and loaded with [32P]orthophosphate in vivo. The cells were then mock-triggered or stimulated for degranulation using DNP-BSA for 20 min. The cells were washed, lysed in Triton X-100, and immunoprecipitations were performed using SNAP-23, syntaxin 4, and syntaxin 3 antisera. The immunoprecipitates were analyzed by SDS-PAGE and analyzed by PhosphorImager (to visualize 32P-labeled proteins) or by immunoblot analysis using the indicated antibodies. The more rapidly migrating band in the SNAP-23 immunoblot is a degradation product of SNAP-23 that is only seen in rat tissues. B. RBL cells were coated with DNP-specific IgE and incubated with BSA (mock-triggered) or DNP-BSA (triggered) for 20 min. The cells were lysed in Triton X-100 and SNAP-23 was isolated by immunoprecipitation (I-P) using a SNAP-23 antiserum. The immunoprecipitates were incubated in alkaline phosphatase buffer in the absence (buffer alone) or presence (phosphatase) of alkaline phosphatase. The samples were then analyzed by immunoblot analysis using a SNAP-23 antibody.

SNAP-23 Phosphorylation and Mast Cell Degranulation Occur with Similar Kinetics—To establish whether the kinetics of SNAP-23 phosphorylation correlate with the kinetics of mast cell degranulation, isolated mast cells were stimulated with a cross-linking reagent and the phosphorylation status of SNAP-23 was determined by SDS-PAGE and PhosphorImager analysis (Fig. 1A). In agreement with published results (15), syntaxin 4 was phosphorylated in mock-treated RBL cells and phosphorylation was not altered by stimulation. Whereas syntaxin 3 is clearly present in RBL cells, syntaxin 3 was not detectably phosphorylated either before or after stimulation. By contrast, SNAP-23, which was also phosphorylated to a small extent in the resting state, underwent a stimulation-dependent increase in phosphorylation after stimulation with DNP-BSA. Under these conditions we observed a 2.5-fold increase in SNAP-23 phosphorylation after 20 min of stimulation as compared with mock-treated cells.

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SNAP-23 Phosphorylation and Mast Cell Degranulation Occur with Similar Kinetics—To establish whether the kinetics of SNAP-23 phosphorylation correlate with the kinetics of mast
Snap-23 is transiently phosphorylated in RBL mast cells. A, RBL cells were sensitized with DNP-specific IgE and incubated with DNP-BSA for various times. At the end of the indicated time point, the cell supernatants were harvested and the amount of β-hexosaminidase released from the cells was calculated. In mock-stimulated cells, we routinely find less than 5% of the total pool of β-hexosaminidase released into the medium. Each data point is a mean ± S.D. of three independent experiments. B and C, RBL cells were coated with DNP-specific IgE and loaded with [32P]orthophosphate in vivo. The cells were then incubated with DNP-BSA (triggered) for various times and washed in ice-cold buffer. The cells were lysed in Triton X-100 and SNAP-23 was isolated by immunoprecipitation using anti-SNAP-23 antisera. An aliquot was also analyzed using preimmune serum to demonstrate the specificity of the immunoprecipitation reaction. The immunoprecipitates were analyzed by SDS-PAGE and analyzed by PhosphorImager to visualize 32P-labeled SNAP-23 or by immunoblot analysis using SNAP-23 antibodies. The intensity of the phospho-SNAP-23 signal was quantitated using PhosphorImager and is shown for the 60-min secretion time course in B and the 20-min secretion time course in C.

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SNAP-23 Is Phosphorylated by Protein Kinase C—Protein kinase C (PKC) is a key kinase involved in mast cell signaling and is an important regulator of exocytosis (10). To determine whether PKC is involved in SNAP-23 phosphorylation, RBL cells were treated with the PKC inhibitor bisindolylmaleimide (I) for 10 min before and during the stimulation. Bisindolylmaleimide (I) inhibits a wide variety of PKC isoforms, including both conventional and non-conventional PKCs (32). Both stimulated exocytosis and SNAP-23 phosphorylation were inhibited by treatment of RBL cells with bisindolylmaleimide (I), revealing that SNAP-23 phosphorylation requires PKC activity in RBL cells (Fig. 3A).

We next set out to determine whether SNAP-23 could serve as a substrate for PKC. In vitro phosphorylation experiments using purified PKC and recombinant GST-SNAP-23 fusion proteins revealed that the latter was phosphorylated by PKC under conditions in which GST alone was not (Fig. 3B). Whereas such an analysis does not prove that PKC is responsible for SNAP-23 phosphorylation in vivo, it prompted us to identify the PKC phosphorylation sites in SNAP-23.

To establish more clearly if PKC was involved in SNAP-23 phosphorylation in vivo, we performed two-dimensional phosphopeptide analyses of endogenous phospho-SNAP-23 isolated from resting RBL cells, stimulated RBL cells, and GST-SNAP-23 phosphorylated by purified PKC in vitro. In the mock-stimulated state, one SNAP-23 phosphopeptide is observed (indicated by the arrowhead in Fig. 3C). Upon stimulation the intensity of this basal spot is not altered, demonstrating that phosphorylation of this phosphopeptide is not augmented or diminished by mast cell triggering for exocytosis. Cross-linking of surface FceRI with DNP-BSA leads to the appearance of three major phosphopeptides (circled in Fig. 3C) and quantitative analysis of multiple maps revealed that these three spots represent 61 ± 6% of the total phosphorylation signal and 75 ± 6% of the induced phosphorylation signal. Comparing the phosphopeptide map of endogenous SNAP-23 from RBL cells to that of in vitro phosphorylated GST-SNAP-23 demonstrated that the “basal” phosphorylation site as well as the three, induced phosphopeptides were generated by PKC treatment, further suggesting that PKC phosphorylates SNAP-23 in vivo. In addition to the highlighted phosphopeptides, PKC directly phosphorylates additional residues on GST-SNAP-23 that are not present in endogenous SNAP-23 isolated from mast cells. In vitro phosphorylation artifacts that are not relevant to in vivo phosphorylation are not uncommon. In this case we did not choose to characterize these “irrelevant” phosphopeptides further.

Identification of SNAP-23 Phosphorylation Sites—Having identified SNAP-23 as a protein that is inducibly phosphorylated by triggering mast cell exocytosis, we set out to identify the phosphorylation sites on the protein. Because phosphorylation of GST-SNAP-23 with PKC resulted in phosphopeptide maps that contained most of the spots present in the maps of endogenous SNAP-23 phosphorylated in RBL cells (Fig. 3B), we individually mutated each of the 29 serine and threonine residues in GST-SNAP-23 to alanine, phosphorylated the recombinant proteins with PKC as a substrate for PKC.

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phosphorylation of an additional residue that was not identified in our initial screen of mutants.

Additional mutagenesis studies revealed that the phosphopeptide indicated by an asterisk in Fig. 4 represents Ser161 (as identified by Polgar et al. (17)). This minor phosphopeptide was not reproducibly observed in vivo, accounted for only 1.1 ± 2% of the induced phosphorylation signal, and was therefore not analyzed further. The phosphopeptide migrating just below and to the left of the basal phosphorylation site represented only 22 ± 10% of the induced phosphorylation signal, however, because this phosphopeptide was not generated by in vitro phosphorylation of GST-SNAP-23 with PKC the nature of this residue was not determined.

To unambiguously demonstrate that SNAP-23 Ser95 and Ser120 are phosphorylated upon mast cell stimulation, we introduced wild-type FLAG-tagged SNAP-23 or its Ser95 and Ser120 mutants into RBL cells and triggered the cells for degranulation. The addition of the FLAG-epitope tag allowed us to distinguish endogenous rat SNAP-23 from the transfected rat SNAP-23 mutants. Phosphopeptide mapping studies confirmed that FLAG-SNAP-23 was inducibly phosphorylated like endogenous SNAP-23 by FceRI cross-linking (Fig. 4). Most

![Phosphorylation of SNAP-23 Regulates Exocytosis from Mast Cells](image-url)
importantly, mutation of Ser95 and Ser120 to Ala also resulted in the disappearance of the three induced phosphopeptide species observed in endogenous SNAP-23. These data demonstrate that Ser95 and Ser120 are the predominant phosphorylation sites of SNAP-23 in RBL cells.

**Generation of Phospho-SNAP-23-specific Antibodies**—Having identified Ser120 and Ser95 as the major phosphorylation sites in SNAP-23 in RBL cells, we generated rabbit antisera against SNAP-23 peptides containing phospho-Ser120 or phospho-Ser95 (Fig. 5A). To confirm the specificity of these antisera, HeLa cells were transiently transfected with cDNAs encoding empty expression vector (mock) or FLAG-tagged wild-type SNAP-23, SNAP-23 S95A, and SNAP-23 S120A as indicated. The cells were stimulated with phorbol myristate acetate and ionomycin for 20 min and aliquots of the cell lysates were analyzed by immunoblotting with a phospho-SNAP-23-Ser120 antiserum (upper panel), a phospho-SNAP-23-Ser95 antiserum (middle panel), or an antibody that recognizes total SNAP-23 (lower panel). Note that FLAG-tagged rat SNAP-23 migrates with a slower electrophoretic mobility than endogenous human SNAP-23 in HeLa cells.

**C**, RBL mast cells and mouse bone marrow-derived mast cells were coated with DNP-specific IgE and mock-stimulated or stimulated with DNP-BSA for 15 min. Equal aliquots of the cell lysates were analyzed by immunoblotting with a phospho-SNAP-23-Ser120 antiserum, a phospho-SNAP-23-Ser95 antiserum, or an antibody that recognizes total SNAP-23.

**Fig. 5.** Phosphorylation site-specific antibodies recognize phospho-SNAP-23 in rat and mouse mast cells. A, a cartoon of the structure of SNAP-23 highlighting the location of Ser95 and Ser120 in relation to the cysteine-rich linker region of SNAP-23 and the amino- and carboxyl-terminal coiled-coil domains. B, HeLa cells were transiently transfected with cDNAs encoding empty expression vector (mock) or FLAG-tagged wild-type SNAP-23, SNAP-23 S95A, and SNAP-23 S120A as indicated. The cells were stimulated with phorbol myristate acetate and ionomycin for 20 min and aliquots of the cell lysates were analyzed by immunoblotting with a phospho-SNAP-23-Ser120 antiserum (upper panel), a phospho-SNAP-23-Ser95 antiserum (middle panel), or an antibody that recognizes total SNAP-23 (lower panel). Note that FLAG-tagged rat SNAP-23 migrates with a slower electrophoretic mobility than endogenous human SNAP-23 in HeLa cells. C, RBL mast cells and mouse bone marrow-derived mast cells were coated with DNP-specific IgE and mock-stimulated or stimulated with DNP-BSA for 15 min. Equal aliquots of the cell lysates were analyzed by immunoblotting with a phospho-SNAP-23-Ser120 antiserum, a phospho-SNAP-23-Ser95 antiserum, or an antibody that recognizes total SNAP-23. (Fig. 5B). Immunoblot analysis with a SNAP-23 antibody confirmed the presence of endogenous human SNAP-23 in transfected FLAG-SNAP-23 in the cells. Note that neither phospho-SNAP-23-specific antibody recognized endogenous HeLa SNAP-23, as Ser120 is not present in human SNAP-23 and the sequence surrounding Ser95 is not conserved between human SNAP-23 and rodent SNAP-23.

The anti-phospho-SNAP-23 antibodies were then used to examine endogenous SNAP-23 phosphorylation in RBL cells and bone marrow-derived mouse mast cells. In unstimulated RBL cells the antisera recognized a very weak phospho-SNAP-23 band (Fig. 5C), likely representing SNAP-23 that is phosphorylated during the low basal degranulation observed in mast cells. In contrast, when cells are stimulated with DNP-BSA for 15 min, very strong phospho-SNAP-23-Ser120 and phospho-SNAP-23-Ser95 signals were observed in RBL cells (Fig. 5C). In addition, when bone marrow-derived mast cells were triggered we observed robust phosphorylation of SNAP-23 using the phospho-SNAP-23-Ser120 antibody (Fig. 5C; Ser95...
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**Fig. 6.** Phospho-SNAP-23-Ser^{120} resides exclusively on membranes. RBL mast cells were sensitized with DNP-specific IgE and mock-stimulated or stimulated with DNP-BSA for 15 min. The cells were fractionated into cytosolic and membrane fractions, and equivalent aliquots of each fraction were analyzed by immunoblotting with phospho-SNAP-23-Ser^{120} antiserum (upper panel), an antibody that recognizes total SNAP-23 (middle panel), or an antibody that recognizes syntaxin 4 (lower panel). In a parallel experiment, RBL cells expressing the cytosolic control protein GFP were fractionated and the cytosolic and membrane fractions were analyzed by immunoblotting with a GFP antibody.

Phosphorylation was not examined. These data demonstrate that endogenous SNAP-23 in both mast cell types is phosphorylated on Ser^{120} upon stimulation of exocytosis. These data highlight the specificity of these antibodies for phospho-SNAP-23 and demonstrate that SNAP-23 phosphorylation is not only induced in RBL cells but in bone marrow-derived mast cells.

**Phosphorylated SNAP-23 Is Bound to Membranes—**Both Ser^{95} and Ser^{120} are adjacent to the cysteine-rich region known to be involved in the anchoring of SNAP-23 to the plasma membrane by palmitoylation (33). To investigate the possibility that SNAP-23 phosphorylation regulates membrane attachment of SNAP-23, we isolated membrane and cytosol fractions from resting and stimulated RBL cells. Both before and after DNP-BSA stimulation, essentially all phospho-SNAP-23-Ser^{120}, like the total pool of SNAP-23 and syntaxin 4, was associated with the membranes (Fig. 6). The membrane fraction was essentially devoid of the cytosolic marker protein GFP, confirming the ability of our subcellular fractionation protocol to separate cellular membranes from cytosol. These data demonstrate that phosphorylation of SNAP-23 does not interfere with membrane attachment of SNAP-23.

**SNAP-23 Is Phosphorylated in Stimulated Mouse Platelets—**It has recently been reported that triggering of platelet degranulation results in SNAP-23 phosphorylation on Ser^{23}/Thr^{24} and Ser^{161} (17). Given our failure to identify these phosphorylation sites in stimulated mast cells together with our identification of Ser^{95} and Ser^{120} as the major induced phosphorylation sites in SNAP-23, we sought to examine SNAP-23 phosphorylation in stimulated mouse platelets. Thrombin activates platelets and induced secretion from each of the three platelet granule stores: α-granules, dense core granule, and lysosomes. Thrombin treatment leads to a time-dependent increase in SNAP-23 phosphorylation as revealed by immunoblotting using phospho-SNAP-23-Ser^{120} and phospho-SNAP-23-Ser^{95} antibodies (Fig. 7). Note that as in mast cells, the characteristic fuzziness of platelet SNAP-23 in the SNAP-23 immunoblot indicates phosphorylation. In this same experiment the extent of release from each of the granules was as follows: 6 and 38% for α-granules, 52 and 73% for dense core granules, and 4 and 27% for lysosomes at the 15- and 120-s time points, respectively. These data demonstrate that as in rodent mast cells, SNAP-23 in mouse platelets is phosphorylated upon stimulation.

**Phosphorylation site-specific antibodies recognize phospho-SNAP-23 in mouse platelets.** Platelets were kept resting or stimulated for the indicated times with 0.5 units/ml thrombin. The reactions were stopped by the addition of hirudin and SDS-PAGE sample buffer to each sample. The SNAP-23 present in each sample was analyzed by immunoblotting with a phospho-SNAP-Ser^{95} antiserum, a phospho-SNAP-23-Ser^{95} antiserum, or an antibody that recognizes total SNAP-23. Thrombin treatment leads to a time-dependent increase in SNAP-23 phosphorylation on Ser^{95} and Ser^{120} following thrombin stimulation.

A Significant Pool of SNAP-23 Is Phosphorylated Upon Stimulation—Whereas we were able to unambiguously determine the induced SNAP-23 phosphorylation sites by phosphopeptide mapping and site-directed mutagenesis studies, such techniques do not readily allow one to determine the extent of protein phosphorylation on each residue. To examine the significance of SNAP-23 phosphorylation, we took advantage of our finding that the phospho-SNAP-23-Ser^{120} antiserum was capable of immunoprecipitating phospho-SNAP-23 from cell extracts. Whereas the phospho-SNAP-23-Ser^{95} antiserum was effective for immunoblot analysis, this antiserum was not effective in removing all phospho-SNAP-23-Ser^{95} from cell extracts. RBL mast cells were triggered with DNP-BSA for 10 min (or not), cell extracts were prepared, and immunoprecipitations of equal aliquots of cell extract were carried out using control rabbit antiserum, an antiserum that recognizes total SNAP-23, or phospho-SNAP-23-Ser^{120} antiserum. Analysis of both the immunoprecipitated material as well as aliquots of the remaining supernatant from each reaction confirmed that the control rabbit antiserum did not nonspecifically bind SNAP-23, and that the pan-SNAP-23 or phospho-SNAP-23-Ser^{120} antisera removed the majority of SNAP-23 or phospho-SNAP-23-Ser^{120} from each reaction, respectively (Fig. 8A). Quantitative analysis revealed that the phospho-SNAP-23-Ser^{120} antiserum bound 2% of the total pool of SNAP-23 in unstimulated cells, and that after stimulation the phospho-SNAP-23-Ser^{120} antiserum bound more than 10% of the total pool of SNAP-23 (Fig. 8B). To eliminate the possibility these values were skewed by co-precipitation of non-phosphorylated SNAP-23 with phospho-SNAP-23, cell lysates were boiled in SDS prior to immunoprecipitation to ensure dissociation of all SNARE proteins. After this treatment the percent of phospho-SNAP-23 present in the SNAP-23 immunoprecipitate was similar to that in the unboiled samples, increasing from 0.2% before stimulation to 8.8% after stimulation. These data reveal that stimulation of mast cell degranulation leads to phosphorylation of a significant fraction of the total pool of SNAP-23.

**Phosphorylation of SNAP-23 Modulates Regulated Exocytosis from RBL Mast Cells—**Given the striking similarity in kinetics of mast cell degranulation and regulated phosphorylation of SNAP-23, we introduced a SNAP-23 phosphorylation mutant into RBL cells to determine whether overexpression of this protein interfered with mast cell degranulation. For these studies RBL cells were transfected with empty FLAG vector alone, FLAG-tagged wild-type SNAP-23, the FLAG-tagged SNAP-23 S95A/S120A double mutant (that is not subject to induced phosphorylation), or the FLAG-tagged SNAP-23 S95D/
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S120D double mutant (that is a phosphomimetic mutant). Each construct was transiently transfected together with a trace amount of human growth hormone secretion reporter as described previously (29). To determine the effect of the mutations on regulated exocytosis, we calculated the percent of secretion from cells expressing each construct (Fig. 9A). Overexpression of wild-type SNAP-23 had no effect on the extent of regulated exocytosis from RBL cells (data not shown). By contrast, overexpression of both SNAP-23 phosphorylation mutants resulted in statistically significant alterations in exocytosis as compared with cells overexpressing wild-type SNAP-23 (measured 15 min after triggering the cells with DNP-BSA). Whereas cells expressing the SNAP-23 Ser/Ala mutants showed a modest inhibition of exocytosis (23% inhibition relative to wild-type SNAP-23), the cells expressing the phosphomimetic Ser/Asp mutant showed a much more profound inhibition of exocytosis (35% inhibition relative to wild-type SNAP-23), the cells expressing the phospho-SNAP-23—S120D double mutant (that is a phosphomimetic mutant). Each construct was transiently transfected together with a trace amount of human growth hormone secretion reporter as described previously (29). To determine the effect of the mutations on regulated exocytosis, we calculated the percent of secretion from cells expressing each construct (Fig. 9A). Overexpression of wild-type SNAP-23 had no effect on the extent of regulated exocytosis from RBL cells (data not shown). By contrast, overexpression of both SNAP-23 phosphorylation mutants resulted in statistically significant alterations in exocytosis as compared with cells overexpressing wild-type SNAP-23 (measured 15 min after triggering the cells with DNP-BSA). Whereas cells expressing the SNAP-23 Ser/Ala mutants showed a modest inhibition of exocytosis (23% inhibition relative to wild-type SNAP-23), the cells expressing the phosphomimetic Ser/Asp mutant showed a much more profound inhibition of exocytosis (35% inhibition relative to wild-type SNAP-23). Immunoblot analysis of the cells confirmed that each form of FLAG-SNAP-23 was expressed in the cells and the level of expression of each of these FLAG-tagged forms of SNAP-23 was approximately twice that of endogenous SNAP-23 (Fig. 9B). Co-immunoprecipitation studies confirmed that each mutant protein was able to interact with syntaxin 4 and no obvious differences in syntaxin binding between FLAG-SNAP-23 and these mutants were detected (data not shown). Because expression of either a SNAP-23 mutant that cannot be phosphorylated or a SNAP-23 mutant that appears constitutively phosphorylated inhibits exocytosis even in cells that constitutively express large amounts of endogenous SNAP-23, these data demonstrate that regulation of SNAP-23 phosphorylation is an important regulator of stimulated mast cell exocytosis.

Syntaxin 4 and VAMP-2 are Preferentially Bound to Phospho-SNAP-23—SNAP-23 is known to bind to both syntaxin 4 and VAMP-2 and all three proteins are important for mast cell degranulation (18–21). We have observed that only a small proportion of SNAP-23 is bound to syntaxin 4 and VAMP-2 in RBL mast cells, and we set out to determine whether triggering exocytosis altered syntaxin 4 or VAMP-2 binding to SNAP-23. Triggering exocytosis for 10 min did not lead to any detectable change in the extent of SNAP-23 binding to syntaxin 4 or VAMP-2, with ~5% of all SNAP-23 binding to syntaxin 4 and VAMP-2 both before and after stimulation. Curiously, we found that after stimulation the majority of SNAP-23 bound to both syntaxin 4 and VAMP-2 was phosphorylated, because very little non-phosphorylated SNAP-23 could be detected in complex with either syntaxin 4 or VAMP-2 (Fig. 10). It should be pointed out, however, that even under these conditions the majority of phospho-SNAP-23 is not bound to syntaxin 4 (and vice versa). These data demonstrate that triggering mast cell degranulation promotes SNAP-23 phosphorylation and leads to the preferential association of syntaxin 4 and VAMP-2 with phospho-SNAP-23. Like SNAP-25 (34), SNAP-23 is unable to efficiently bind to VAMP in the absence of syntaxin family members, suggesting that the preferred, but not exclusive, substrate for SNAP-23 phosphorylation may be the SNAP-23 present in the ternary SNARE complex.

DISCUSSION

Secretory granule fusion with the plasma membrane is the final step in a complex series of biochemical events that lead to the release of inflammatory mediators from mast cells. As in membrane/membrane fusion events in other cell types, members of the SNARE fusion machinery are thought to mediate regulated exocytosis in mast cells. SNAP-23 and syntaxin 4 have been proposed to be essential plasma membrane SNAREs (18–20), whereas on the secretory granules themselves VAMP-2, VAMP-7, and VAMP-8 have been implicated in the granule/plasma membrane fusion process (18, 19, 21, 35). In this study we have investigated changes in SNAP phosphorylation during mast cell exocytosis and studied the biochemical and functional consequences of SNAP phosphorylation in vivo. We find that SNAP-23 is phosphorylated in the RBL mast cell line and in bone marrow-derived mast cells upon FceRI receptor cross-linking, the physiological trigger for mast cell

* N. Puri and P. A. Roche, manuscript in preparation.
degranulation. Using site-directed mutagenesis and phosphopeptide mapping from cells labeled in vivo we have identified Ser95 and Ser120 as the two major phosphorylation sites of SNAP-23 in RBL mast cells, representing 75% of the induced SNAP-23 phosphorylation. These residues are located in the palmitoylated “linker” region separating the amino- and carboxyl-terminal coiled-coil domains of SNAP-23. Whereas Ser95 is conserved in both rodent and human SNAP-23, Ser120 is present in rodent, but not human, SNAP-23. Like syntaxin 4 in platelets (24) and RBL cells (Ref. 15 and this study), SNAP-23 is constitutively phosphorylated in unstimulated cells. Phosphopeptide mapping studies using SNAP-23 phosphorylation mutants confirmed that the basal phosphorylation site is not Ser95 or Ser120, therefore unambiguous determination of the basal phosphorylation site(s) in SNAP-23 will require additional studies.

In our attempt to reveal a biological consequence of SNAP-23 phosphorylation, we overexpressed SNAP-23 phosphorylation mutants into RBL mast cells together with a human growth hormone secretion reporter plasmid as described in the text. The cells were sensitized with DNP-specific IgE and mock-stimulated or stimulated with DNP-BSA. Cell supernatants were harvested after 15 min and the extent of degranulation was determined by measuring the amount of human growth hormone released from the cells. The amount of secretion from cells in each experimental condition was expressed as a percentage with DNP-BSA. Cell supernatants were harvested after 15 min and the extent of degranulation was determined by measuring the amount of hormone secretion reporter plasmid as described in the text. The cells were sensitized with DNP-specific IgE and mock-stimulated or stimulated with FLAG-tagged wild-type SNAP-23, SNAP-23 S95A/S120A, or SNAP-23 S95D/S120D that were mock-stimulated or stimulated with DNP-BSA were analyzed by immunoblot analysis using a SNAP-23 carboxyl terminus antibody. This antibody detects endogenous SNAP-23 and FLAG-SNAP-23 equally well. The mobility of endogenous rat SNAP-23 and transfected FLAG-SNAP-23 are indicated by arrows. A representative blot is shown.

Most studies using in vitro phosphorylated SNAP-23 mutants have suggested that phosphorylation inhibits SNARE interactions (13–17). By contrast, we find that after stimulation essentially all of the SNAP-23 bound to syntaxin 4 and VAMP-2 is phosphorylated, clearly demonstrating that in vivo phosphorylation of SNAP-23 does not prevent its binding to these SNAREs. Curiously, we find that despite the preferential association of phospho-SNAP-23 with syntaxin 4 and VAMP-2, stimulation does not alter the absolute amount of SNAP-23 bound to these proteins. Indeed, co-immunoprecipitation experiments revealed that a large proportion of phospho-SNAP-23 is not bound to syntaxin 4, a finding that is consistent with our failure to detect changes in the total amount of SNAP-23 bound to syntaxin 4 after stimulation. Quantitative analyses revealed that in RBL mast cells only 5% of all SNAP-23 is bound to syntaxin 4 or VAMP-2 and less than 2% of syntaxin 4 is bound to SNAP-23 both before and after stimulation, demonstrating that the vast majority of SNAP-23, syntaxin 4, and VAMP-2 are either “free” or bound to other proteins (such as Munc18c (21)). We have found that, like SNAP-25 (34), VAMP-2 is unable to efficiently bind to SNAP-23 in the absence of syntaxin 4. This is consistent with the idea that the SNAP-23 present in the ternary SNARE complex is the substrate for phosphorylation, however, additional studies will be required to examine this in greater detail.

During the course of our investigation of SNAP-23 phosphorylation in rodent mast cells Polgar et al. (17) published a study showing that SNAP-23 Ser23/Thr24 and Ser161 were phosphorylated when human platelets were activated. We found that while rat SNAP-23 Ser161 was very efficiently phosphorylated by protein kinase C in vitro, quantitative analysis of multiple phosphopeptide maps revealed that phosphorylation of SNAP-23 Ser161 represented only 1% of induced SNAP-23 phosphorylation. Furthermore, we did not observe differences in phosphorylation of a FLAG-tagged SNAP-23 Ser23/Thr24 mutant relative to wild-type FLAG-tagged-SNAP-23 by one-dimensional phosphopeptide mapping. We did examine the phosphorylation of activated mouse platelets using our phosphorylation state-specific antibodies and these studies clearly showed that SNAP-23 Ser95 and Ser120 are phosphorylated when mouse platelets are activated with thrombin. It is interesting to note that Polgar et al. (17) also observed SNAP-23...
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A

**Immunoprecipitate**

- Syntaxin 4
- VAMP-2

**Immuno blot**

- control
- SNAP-23
- SNAP-23 (S120P)

**mock**

**DNP-BSA**

B

**SNAP-23-Ser120**

| mock | DNP-BSA |
|------|---------|
| Syntaxin 4 | 10% | 20% |
| VAMP-2 | 20% | 30% |

**Fig. 10.** After mast cell stimulation most syntaxin 4- and VAMP-2-associated SNAP-23 is phosphorylated. A, RBL mast cells were sensitized with DNP-specific IgE and mock-stimulated or stimulated with DNP-BSA for 10 min. The cells were lysed in Triton X-100 and equal portions of each lysate were analyzed by immunoprecipitation using a control (preimmune) antiserum, an antiserum that recognizes total SNAP-23, or an antiserum that recognizes phospho-SNAP-23-Ser^{120}. Aliquots of each immunoprecipitate were analyzed by SDS-PAGE and immunoblotting using antibodies that recognize rat syntaxin 4 (upper panels) or VAMP-2 (lower panels). B, the percent of all SNAP-23-syntaxin 4 complexes containing phospho-SNAP-23-Ser^{120} was calculated by expressing the amount of syntaxin 4 in a phospho-SNAP-23-Ser^{120} immunoprecipitate relative to the total amount of syntaxin 4 bound to all SNAP-23 in the sample. Each data point represents a mean \( \pm \) S.D. of four independent experiments. The percent of all SNAP-23-VAMP-2 complexes containing phospho-SNAP-23-Ser^{120} was calculated by expressing the amount of VAMP-2 in a phospho-SNAP-23-Ser^{120} immunoprecipitate relative to the total amount of VAMP-2 bound to all SNAP-23 in the sample. Each data point represents a mean \( \pm \) S.D. of two independent experiments. Analysis of the supernatant after each immunoprecipitation confirmed that the SNAP-23 antibody and phospho-SNAP-23-Ser^{120} antibody removed essentially all SNAP-23 and phospho-SNAP-23-Ser^{120} from the sample, respectively.

Ser^{120} phosphorylation in vitro but did not identify the in vivo phosphopeptide by mass spectrometry. Whether the differences between the phosphorylation sites identified by us and by Polgar et al. (17) represent quantitative differences in the extent of phosphorylation of particular residues or differences between rodent platelets and human platelets remains to be resolved. Nevertheless, it is clear from our data that SNAP-23 Ser^{120} and Ser^{120} are the dominant inducible SNAP-23 phosphorylation sites in rodent mast cells and platelets.

It is interesting to compare and contrast the phosphorylation of SNAP-23 with that of its neuronal homolog SNAP-25. SNAP-25 is phosphorylated on Ser^{187} when neuroendocrine cells (or pancreatic beta cells) are directly stimulated with phorbol myristate acetate (16, 36, 37), and phosphorylation of SNAP-25 alters binding to syntaxin in vitro (16). However, Ser^{187} is located in one of the coiled-coil domains known to regulate SNAP-25/syntaxin association, so in this respect an effect on SNARE binding is not unexpected. In marked contrast to what we and others have observed with respect to SNAP-23 phosphorylation (this study and Ref. 17), the kinetics of SNAP-25 phosphorylation do not correlate with the kinetics of exocytosis either in regulated secretion from PC12 cells (37) or pancreatic beta cells (36). We find that inhibiting PKC activity with bisindolylmaleimide (I) prevents regulated SNAP-23 phosphorylation and mast cell exocytosis, whereas bisindolylmaleimide (I) prevented stimulus-induced SNAP-25 phosphorylation but had no effect on triggered exocytosis from either PC12 cells (37) or pancreatic beta cells (36). Such data leave the importance of SNAP-25 phosphorylation in regulated exocytosis an open question. Whereas SNAP-25 phosphorylation has clearly been observed when PC12 cells are cultured in nerve growth factor (38), when seizure activity and LTP are induced in hippocampal neurons (39), and when mice are chronically administered morphine (40), there is no direct evidence that these changes are a consequence of stimulated exocytosis. Indeed, we were unable to demonstrate phosphorylation of SNAP-25 in PC12 cells that were stimulated using physiological triggers for degranulation (41).

Kinetic analyses revealed a correlation between stimulus-induced mast cell degranulation and SNAP-25 phosphorylation, although whether phosphorylation occurs immediately prior to membrane fusion, concomitant with fusion, or immediately following membrane fusion could not be resolved by the techniques used here. Although we are unable to precisely identify the molecular mechanism by which SNAP-23 phosphorylation regulates exocytosis, we currently favor the view that phosphorylation primarily affects a post-fusion step in exocytosis, such as SNARE recycling. This is based primarily on our assumption that if phosphorylation were a prerequisite for exocytosis, then preventing phosphorylation (by overexpressing the SNAP-23 S95A/S120A phosphorylation mutant) would inhibit exocytosis more than simply introducing a phospho-mimetic mutant (which could even augment exocytosis). This was not the case, suggesting that the two classes of phosphorylation mutants inhibited exocytosis by different mechanisms. Phosphorylation of SNAP-23 Ser^{55}/Ser^{120} is clearly important for exocytosis, as demonstrated by our results using the SNAP-23 S95A/S120A mutant. This is consistent with a vast literature on the importance of protein phosphorylation on function in a variety of systems. On the other hand, we have shown that after an initial burst of phosphorylation, SNAP-23 becomes dephosphorylated. Dephosphorylation could also have a specific role in exocytosis. If SNAP-23 dephosphorylation enhances SNARE complex disassembly, then overexpression of the phosphomimetic SNAP-23 S95D/S120D mutant could limit the recycling of SNAREs, thereby inhibiting exocytosis. There is clear evidence that efficient SNARE disassembly/reuse is important for exocytosis, as revealed in the phenotype of the temperature-sensitive mutations of NSF in *Drosophila* (42) and by introducing ATPase-deficient forms of NSF into mast cells (29). In both cases, inactivation of the SNARE disassembly enzyme, NSF, leads to an accumulation of SNARE complexes and to a cessation of regulated exocytosis. Consistently, our preliminary studies revealed that the absolute amount of SNAP-23 complexes present before or after stimulation was very slightly higher in RBL cells overexpressing the SNAP-23 phosphomimetic mutant, a result that is consistent with this hypothesis (data not shown).

Whereas we did not observe dramatic alterations in the absolute amount of SNAP-23 bound to either syntaxin 4 or VAMP-2 when either SNAP-23 phosphorylation mutant was introduced into RBL mast cells, we did observe the selective inclusion of phospho-SNAP-23 into SNAP-23-syntaxin 4-VAMP-2 complexes after stimulation. While we favor a model in which triggering exocytosis stimulates the preferential phosphorylation of SNARE complex-associated SNAP-23, we cannot formally rule out the possibility that free SNAP-23 becomes phosphorylated and then displaces non-phosphorylated SNAP-23. In fact, although after stimulation most SNAP-23 bound to syntaxin 4 and VAMP-2 is phosphorylated, most phospo-
SNAP-23 is not necessarily bound to syntaxin 4 or VAMP-2. Such an observation highlights the difficulty in identifying a specific molecular mechanism to explain the inhibition of exocytosis observed here, because only a small amount of SNAP-23, syntaxin, or VAMP are actually present in SNARE complexes at any given time. Although further analysis will be required to determine the precise mechanistic rationale, it is clear that phosphorylation of SNAP-23 is important for efficient regulated exocytosis from mast cells.

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