Phosphorylation of SNAP-23 regulates its dynamic membrane association during Mast Cell exocytosis

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Summary Statement
The current study has revealed the phosphorylation dependent dynamic nature of membrane association of SNAP-23 for mediation of different fusion steps in compound exocytosis from mast cells during allergen challenge.
Abstract

Mast cells (MCs) on allergen challenge, respond by release of pre-stored mediators from their secretory granules by transient mechanism of porosome-mediated cell secretion. The target-SNARE SNAP-23 has been shown to be important for MC exocytosis and our previous studies revealed presence of one basal (Thr\textsuperscript{102}) and two induced (Ser\textsuperscript{95} and Ser\textsuperscript{120}) phosphorylation sites in its linker region. To study the role of SNAP-23 phosphorylation in the regulation of exocytosis, Green fluorescence protein-tagged wildtype SNAP-23 (GFP-SNAP-23) and its phosphorylation mutants were transfected into RBL-2H3 MCs. Studies on GFP-SNAP-23 transfected MCs revealed some dynamic changes in SNAP-23 membrane association. SNAP-23 was associated with plasma membrane in resting MCs, however on activation, a portion of it translocated to cytosol and internal membranes. These internal locations were secretory granule membranes. This dynamic change in the membrane association of SNAP-23 in MCs may be important for mediating internal granule-granule fusions in compound exocytosis. Further studies with SNAP-23 phosphorylation mutants revealed an important role for the phosphorylation at Thr\textsuperscript{102} in its initial, and of induced phosphorylation at Ser\textsuperscript{95} and Ser\textsuperscript{120} in its internal, membrane association, during MC exocytosis.

Introduction

MCs are specialized secretory cells that play a crucial role in inflammation and allergic responses (Kalesnikoff and Galli, 2008). During hypersensitivity reactions they are mainly activated by cross-linking of FcεRI bound IgE by a multivalent allergen (Kraft and Kinet, 2007). This physiological trigger initiates a cascade of events that results in the translocation, docking, and fusion of secretory granule with plasma membrane leading to release of inflammatory mediators stored in the secretory granules (Galli et al., 2008; Puri and Roche, 2008). This process proceeds through a transient mechanism of fusion and release called “kiss and run” and cavi capture for a large proportion of granules in mast cells (Balseiro-Gomez et al., 2016). Further, this secretion involves compound exocytosis where either the vesicles fuse with each other prior to plasma membrane fusion (multivesicular exocytosis) or in a sequential manner i.e., one after another underneath the plasma membrane (sequential exocytosis) (Lorentz et al., 2012; Pickett and Edwardson, 2006). Recently it has also been shown that in mast cell the granule fusion happens through a supramolecular complex called porosome at plasma membrane (Balseiro-Gomez et al., 2016; Deng et al., 2010; Hammel and Meilijson, 2012; Jena, 2012).

A class of proteins termed SNAREs (Soluble NSF attachment protein receptor) is known regulator of membrane fusion events involved in exocytosis (Jahn and Sudhof, 1999; Lin and Scheller, 2000). Distinct SNARE proteins on vesicles [termed as vesicle SNAREs (v-SNAREs)] function by linking up with their cognate SNAREs present on target membranes [termed as target-SNARE (t-SNAREs)]. For membrane fusion to occur, the three SNARE proteins from opposing membranes come together to form the minimally required ternary SNARE complex. This involves hydrophobic interactions in the coiled coil domains of SNARE proteins, and is the essential step in membrane fusion (Poirier et al., 1998; Weber et al., 1998). In a secretory cell, one granule may fuse with another granule to grow into a bigger granule or with the plasma membrane for exocytosis, by formation of a circular rosette made up of ternary SNARE complexes. The pairing of this SNARE rosette with plasma membrane is the site defining the fusion pore or porosome where vesicles dock and fuse (Hammel and Meilijson, 2012; Moon et al., 2014).
Formation of trans-SNARE complex and its temporal and spatial regulation has been studied in MCs (Puri and Roche, 2006). The SNAREs must remain inactive in resting MCs, but on allergen challenge, they function rapidly for membrane fusion and degranulation (Hepp et al., 2005). Very little is known about the SNARE function in various fusion steps during exocytosis in MCs. SNAP-23 (Synaptosomal associated protein of 23 kDa) plays a key role during regulated exocytosis (Hepp et al., 2005). Our previous studies have shown that SNAP-23 has a basal phosphorylation site at Thr$^{102}$ (Hepp et al., 2005). It is also transiently phosphorylated during regulated exocytosis and the kinetics of phosphorylation is similar to the kinetics of exocytosis (Hepp et al., 2005). But the precise molecular mechanisms of SNAP-23 function are not known i.e., what happens immediately after receiving a trigger in a sensitized MC during regulated exocytosis. The early induced phosphorylation of SNAP-23 occurs at two sites (Ser$^{95}$ and Ser$^{120}$) close to the cysteine residues thought to be important for membrane association of SNAP-23. So in this study we decided to check SNAP-23 localization in MCs in resting stage, and immediately after receptor cross linking to explore any other important changes that coincide with early transient induced phosphorylation of SNAP-23 and an initial burst of mediator release in response to a physiological trigger (Hepp et al., 2005). While in the past, one report using permeabilized MCs suggested relocation of SNAP-23 to lamellipodia like structures (Guo et al., 1998), no study till date has explored SNAP-23 localization under physiological conditions of an allergen challenge leading to receptor cross linking.

In the current study as a model system for activation of MCs we have used cross linking of DNP-specific IgE sensitized RBL-2H3 MCs with the multivalent antigen Dinitrophenyl-bovine serum albumin (DNP-BSA), exactly as on MCs of atopic individuals during an allergen challenge (Gould et al., 2003). We found that SNAP-23 is associated with plasma membrane in resting MCs but after MCs’ activation, it now moves to internal locations. Then to study if phosphorylation of SNAP-23 has any role in this aspect, mutants of SNAP-23 that cannot be phosphorylated, or are phospho mimetic, cloned in EGFP vector for better visualization, were used. This is the first report that highlights the dynamic nature of SNAP-23 membrane association by showing that SNAP-23 changes its location in MCs immediately after receiving a physiological trigger. The study also goes on to reveal the importance of SNAP-23 basal and induced phosphorylation in its initial membrane association, and the dynamic relocations to internal sites on MC activation, respectively. Further elucidation of this pathway of SNAP-23 trafficking would help to generate a comprehensive view of the complex membrane fusion processes that occur in MCs during exocytosis.

Results

Activation of RBL MCs by IgE cross-linking partially moves SNAP-23 from plasma membrane to intracellular locations

In the present study primed RBL-2H3 MCs were stimulated for degranulation by cross-linking of FcεRI (Hepp et al., 2005). The extent of MC degranulation was measured as β-hexosaminidase release at different time points as shown in Fig. S1A. The degranulation reached a peak between 5-15 min. Using anti-phospho-SNAP-23-Ser$^{120}$ ab (Fig. S1B). It was shown that phosphorylation of SNAP-23 also reached peak at 5 min and it persists till 20 min, indicating that phosphorylation of SNAP-23 is an early and transient event during MC exocytosis (Fig. S1C). Therefore, to capture early changes, all further studies were carried out 10 min after receptor crosslinking. Further, to check whether IgE cross linking affects the SNAP-23 synthesis/amount we quantified the total SNAP-23 pool at resting, IgE sensitized and allergen cross-linked stages of MC by western blotting (for endogenous SNAP-23) and flow cytometry (for EGFP-SNAP-23 transfected RBL MCs) and found no significant difference in the amount of SNAP-23 in any of the stages (Fig. S1D &E). In order to study if these early events lead to any
changes in membrane localization of t-SNARE SNAP-23 during MC activation, localization of SNAP-23 was studied by confocal fluorescence microscopy. Endogenous SNAP-23 as well as transfected GFP-tagged SNAP-23 was analyzed. Visualization by fluorescence confocal microscopy of the endogenous SNAP-23 (by staining with a SNAP-23 specific antibody, data not shown) and the transfected GFP SNAP-23 revealed a smooth pattern of plasma membrane localized SNAP-23 in different Z-sections of IgE sensitized MCs (Fig. 1A). But, 10 min after cross-linking the FcεRI, the cells appeared flatter, and the plasma membrane showed ruffles (Fig. 1B, DIC image). It can now be seen that SNAP-23 now localized to these plasma membrane ruffles and to some extent on some internal structures (shown by white arrow heads in Fig. 1B). The endogenous and transfected GFP-SNAP-23 were found to behave in similar fashion both before and after MC exocytosis in terms of their membrane localization (data not shown). Further to investigate if the internal locations to which SNAP-23 relocated upon IgE cross-linking, were internal organelle membranes or cytosolic locations, we performed membrane/cytosol fractionation of resting, IgE sensitized, and IgE cross-linked GFP-SNAP-23 transfected as well as untransfected RBL MCs. Quantitative analysis of western blots of the membrane cytosol fractions showed that most (87±2.7% for endogenous and 90±1.5% in case of transfected GFP-SNAP-23) of the SNAP-23 was associated with membrane in resting and (89±2.7% for endogenous and 86±1.5% in case of transfected GFP-SNAP-23) IgE sensitized MCs, however, a small but significant decrease in membrane association (74±2.7% endogenous and 73±2% transfected) of SNAP-23 was observed in MCs 10 min after FcεRI cross-linking (Fig. 1C & D). From this biochemical analysis it is now clear that SNAP-23 relocates to internal cellular locations. Many of the internal membranes to which SNAP-23 relocated 10 min after receptor cross-linking in MCs were spherical, reminiscent of secretory granules, so we decided to investigate the nature of internal membranes by real time live cell microscopic study of GFP-SNAP-23-transfected RBL MCs. These transfected MCs were sensitized with anti-DNP-IgE and lysosomes were marked with Lysotracker Red dye to follow their fate during exocytosis. Before observation in a live cell imaging system one set of cells were mock stimulated (termed as IgE sensitized) and the other one was stimulated with DNP-BSA. The movie capture was started 2 min after mock or allergen stimulation. The cells were then observed for 10 min thereafter. Snap shots were extracted from a representative 10 min movie (supplementary movie 1A&B) obtained from the live cell imaging system. A good staining of lysotracker dye was observed in both the cases. Like our confocal microscopic study, at IgE sensitized stage (during mock stimulation) a smooth plasma membrane staining of SNAP-23 (in green) and internal lysosome staining (in red) was seen. During mock stimulation, the IgE sensitized cells showed slight Brownian movement throughout the 10 min video (Fig. 1E, three time points are shown). But, after allergen addition various dynamic changes in plasma membrane and in lysosomal compartments were seen. At 0 min (which is actually 2min after stimulation) membrane ruffles can be seen. SNAP-23 is associated with them. Some SNAP-23 starts associating with round vesicles [Fig. 1B, (0 min)]. SNAP-23 associated granules harboring lystotracker started appearing at 0 min (2 min after allergen challenge) and they were tracked till 5 min after allergen addition. These vesicles were found to translocate towards plasma membrane (denoted with white arrow and arrow head). In allergen stimulated cells a bunch of granules with SNAP-23 associated membrane gradually appeared at around 4min. These green granules were first seen to translocate inside the cell and then started to queue up towards the plasma membrane. During this process at around 7 min some green granules were seen fusing with each other. Ultimately (at 10 min) almost all granules with SNAP-23 ended up in a bigger granule most probably by homotypic fusion. So, this real time imaging shows movement of SNAP-23 to internal vesicle membranes which may be lysosomal in nature and fusion of some of these vesicles probably during compound exocytosis from MCs on allergen challenge.
SNAP-23 moves from plasma membrane to internal lysosomal membranes during regulated exocytosis of MCs

To confirm the observation obtained from previous experiments we then performed immunofluorescence and confocal microscopy. We have studied the association of SNAP-23 with different internal organelles like Golgi apparatus, Trans Golgi network (TGN), and also late endosome/lysosomes. RBL MCs were first stained with SNAP-23 specific antibody and then counter stained with TGN38 [a type I integral membrane protein primarily localized to the TGN (Humphrey et al., 1993)] and GM130 [Golgi matrix protein of 130 kDa, peripherally associated with the cis-compartment (Nakamura et al., 1997)] specific antibodies at IgE sensitized and receptor cross-linked states (Fig. 2A & B respectively). The confocal microscopy images from Fig. 2A and 2B showed that SNAP-23 is not associated with Golgi and Trans Golgi network at IgE sensitized states of MCs (P. coefficient 0.001± 0.002 & 0.0357± 0.0014 respectively, Fig. 2D) and also after receptor cross-linking it does not relocate to these internal organelle membranes (P. coefficient 0.054± 0.002, Fig. 2D). In order to investigate if any SNAP-23 localized to late endosome/lysosomal membranes in activated MCs, LAMP-3 lysosomal membrane marker in MCs was used. GFP-SNAP-23 transfected MCs were counterstained with anti-LAMP-3 antibody. As shown in Fig. 2C, in IgE sensitized GFP-SNAP-23 expressing MCs SNAP-23 is mainly localized to plasma membrane and LAMP-3 staining is completely internal and negligible colocalization is seen between the two (P.Coefficient 0.17±0.001, Fig. 2D). But after receptor cross-linking, transfected GFP-SNAP-23 was found on plasma membrane ruffles and also on internal membranes, a large number of which also showed staining for LAMP-3 (Fig. 2C). So, a very high level of colocalization was obtained between GFP-SNAP-23 and LAMP-3 (P.Coefficient 0.7±0.03, Fig. 2D). This indicated that 10 min after receptor cross-linking SNAP-23 relocated to internal membranes in MCs, which were LAMP-3 positive and hence lysosomal in nature.

SNAP-23 relocates to secretory granule membranes during regulated exocytosis in MCs.

Since lysosomes in MCs also have secretory functions, and are therefore referred as secretory lysosomes (Puri et al., 2003; Puri and Roche, 2008), so we wanted to check whether SNAP-23 relocates to secretory granule also. Rodent MCs are known to harbor serotonin in granules that are lysosomal in nature, and there is a regulated release of serotonin in response to a physiological trigger (Puri and Roche, 2008). So we decided to locate serotonin cargo in GFP-SNAP-23 transfected RBL cells by counterstaining serotonin with serotonin specific antibody in IgE sensitized and allergen activated RBL MCs. In the IgE sensitized MCs, SNAP-23 showed plasma membrane association and serotonin showed a punctate staining pattern in intracellular organelles (Fig. 3A upper panel) with negligible or no co-localization (0.1±0.018, Fig. 3C). But 10min after FcεRI cross-linking, as SNAP-23 localization pattern changed, it showed partial co-localization with serotonin [P.Coefficient 0.43±0.03 (internal, i.e., excluding plasma membrane) P.Coefficient 0.54±0.018 (including plasma membrane, total)] (Fig. 3A, lower panel). The white arrows and inset image indicate serotonin punctae surrounded by organelle membranes having green GFP-SNAP-23 staining. This indicated that SNAP-23 colocalized to internal secretory granule membranes in MCs on activation by FcεRI cross-linking. Besides, from literatures it is known that t-SNARE STX-3 resides in secretory granule membrane (Puri et al., 2003). So we also looked at STX-3 co-localization, if any, with SNAP-23 in GFP-SNAP-23 transfected RBL MCs by counterstaining these cells with anti-STX-3 ab. In case of IgE sensitized MCs STX-3 is seen on internal granule membranes, and does not show any co-localization with GFP SNAP-23 (Fig. 3B, upper panel; P. coefficient 0.26±0.03, Fig. 3C) expressed on plasma membrane. After receptor cross linking, the relocated SNAP-23 showed very high co-localization with STX-3 on almost circular internal secretory granule membranes (Fig. 3B, lower panel; P. coefficient 0.77±0.02, Fig. 3C). Together these two results indicate that immediately after receptor crosslinking, SNAP-23 relocates to STX-3 harboring internal organelles which may also enclose the secretory granule cargo like Serotonin, and hence are secretory granules.
Phosphorylation of SNAP-23 is important for its membrane association in RBL MCs

SNAP-23 lacks transmembrane domain and is thought to associate with plasma membrane through palmitoylation of its conserved cysteine residues present in the linker region [(Vogel and Roche, 1999) and unpublished data]. Our previous studies have identified one basal phosphorylation site, Threonine^{102}, close to these cysteines in the linker region of SNAP-23. To explore if phosphorylation of T102 has any role in association of SNAP-23 with membranes, T was mutated to A, so that it can no longer be phosphorylated (Fig. 4A). GFP-SNAP-23 T102A transfected RBL MCs were either mock stimulated or receptor crosslinked for 10 min and subjected to membrane cytosol fractionation. Immunoblotting of membrane cytosol fractions of mock stimulated cells revealed significantly lower association (55-60% decrease) of SNAP-23 T102A mutant with membrane in comparison to wildtype SNAP-23 (Fig. 4B & C). Similar results were obtained for transfected cells activated by receptor crosslinking, with a major portion of mutant SNAP-23 residing in the cytosol (60% in cytosol) (Fig. 4B & C).

Further, the induced phosphorylation sites at S^{95} and S^{120} are also very close to the conserved cysteines in the linker region of SNAP-23. To investigate the role of SNAP-23 transient phosphorylation followed by dephosphorylation 20 min after receptor crosslinking in membrane localization of SNAP-23, two kinds of mutants, GFP SNAP-23 S95AS120A (phospho negative, that cannot be phosphorylated) and GFP SNAP-23 S95DS120D (phospho mimetic, constitutively phosphorylated) (Hepp et al., 2005), were used. The amino acid sequence comparison for these two mutants with wildtype SNAP-23 is shown in Fig. 5A. In case of IgE sensitized RBL MCs, SNAP-23 S95A/S120A mutant was shown, by membrane cytosol fractionation, to be mainly associated with membrane, though there was a small but significant decrease in membrane association in comparison to wildtype SNAP-23 (decrease from 90 to 73%) (Fig. 4B & C). In the receptor crosslinked stage, the membrane association of SNAP-23 S95A/S120A mutant showed a drastic decrease in comparison to wildtype SNAP-23 (from 73 to 47%), and a major portion of it was cytosolic (Fig. 4B & C).

The phospho mimetic SNAP23 S95D/S120D mutant mimics the transient phosphorylated state which lasts 5 to 20 mins after receptor crosslinking. In mock stimulated stage, membrane cytosol fractionation of SNAP-23 S95D/S120D transfected RBL cells revealed much lower association of SNAP-23 mutant with membrane in comparison to wildtype SNAP-23 is seen (56% in comparison to 90%) (Fig. 4B & C). After activation, there is a further decrease in membrane association, and increase in cytosolic localization, of SNAP-23 S95D/S120D mutant (43% membrane association) (Fig. 4B & C).

Role of SNAP-23 phosphorylation in its dynamic association with internal membranes during secretory response of MCs

As previous experiments with wildtype SNAP-23 had shown relocation to internal lysosomal membranes on receptor crosslinking, the co-localization of SNAP-23 T102A with lysosomal compartments was also investigated by microscopy after counterstaining transfected cells with LAMP-3 specific antibodies. As shown in Fig. 5A, a lot of intracellular or cytosolic location for transfected SNAP-23 T102A mutant in both IgE sensitized and receptor crosslinked stage was confirmed. SNAP-23 T102A mutant also did not show any co-localization with LAMP-3 in resting stage (P. coefficient 0.17±0.03), as the wildtype SNAP-23 (P. coefficient 0.12±0.25). But even after receptor crosslinking SNAP-23 T102A does not show any significant co-localization with LAMP-3 positive membranes (P. coefficient 0.28±0.06). These results indicate that basal phosphorylation of SNAP-23 at T^{102} may be important for initial association of SNAP-23 with membranes as the membrane association showed a marked decrease even in mock stimulated or resting stage (Fig. 5A). The phosphorylation of T102 may also be important for relocation of SNAP-23 to internal lysosomal membranes on receptor crosslinking as SNAP-23 T102A mutant does not show association with lysosomal membranes on activation and remains stuck in cytosol (Fig. 5D & Table-1).
The increased cytosolic localization of SNAP-23 S95A/S120A mutant was confirmed by immunofluorescence microscopic study. When the co-localization of this SNAP-23 S95A/S120A mutant with lysosomal compartments in resting or activated MCs was tested, by staining transfected MCs with LAMP-3 Ab, no co-localization of SNAP-23 S95A/S120A mutant with LAMP-3 was seen in either stage (P. coefficient 0.20±0.05 & 0.19±0.09 in IgE sensitized and receptor crosslinked MC respectively) (Fig. 5B and Table-1). This study with SNAP-23 S95A/S120A mutant, that cannot be phosphorylated, reveals that transient phosphorylation of SNAP-23 at Ser195 and Ser120 may be important for relocation of SNAP-23 to internal lysosomal membranes, as lack of phosphorylation prevented that and left SNAP-23 stuck in the cytosol on MC activation.

The increased cytosolic association of SNAP-23 S95D/S120D mutant was also observed by confocal fluorescence microscopy. Further when the transfected RBL MCs were co-stained with LAMP-3 Ab, a very high colocalization of SNAP-23 S95D/S120D mutant with LAMP-3 was observed (P. coefficient 0.57±0.02), even in mock stimulated stage. This showed that a large proportion of mutant SNAP-23 was already associated with internal lysosomal membranes rather than with plasma membrane as happens in case of wildtype SNAP-23. On receptor crosslinking, the SNAP-23 S95D/S120D mutant maintains very high co-localization with LAMP-3 (P. coefficient 0.62±0.06) (Fig. 5C and Table-1). These results indicate that the transient induced phosphorylation of SNAP-23 at Ser95 and Ser120 plays a very important role in movement of SNAP-23 to internal locations and then its association with lysosomal membranes.

Role of SNAP-23 phosphorylation in MC exocytosis
SNAP-23 has been shown to play an important role in MC degranulation (Hepp et al., 2005). After observing dramatic effects of mutations in basal and induced phosphorylation sites of SNAP-23 on its membrane association in resting, and activated MCs, it was decided to check the effects of these mutations on MC degranulation. For this, the hGH secretion reporter assay (already discussed in (Puri et al., 2003) and briefly in materials & methods) was performed. The GFP-SNAP-23 phospho mutants and hGH DNA were co-transfected in RBL MCs and their extent of hGH release was assayed at 45 min. In the case of MCs transfected with GFP-SNAP-23 T102A mutant, significant reduction in secretion (~17% reduction in comparison to wildtype SNAP-23) was seen after allergen cross linking (Fig. 6A). In case of the induced phosphorylation site mutations, hGH release assay of GFP-SNAP-23 S95A/S120A and GFP-SNAP-23 S95D/S120D mutant transfected MCs (separately) showed reduction in secretion (~9% and ~19% reduction respectively in comparison to wildtype SNAP-23) after allergen cross linking (Fig. 6A). The expression of transfected GFP-SNAP-23 wildtype and mutants was also compared to that of endogenous SNAP-23 by immunoblotting the lysates from transfected cells with anti-SNAP-23 Ab (Fig. S2A). In all cases, the level of expression of transfected proteins was about 40% of that of endogenous SNAP-23 (Fig. S2B).

Discussion
The plasma membrane t-SNARE SNAP-23 plays an important role in MC exocytosis (Hepp et al., 2005). SNAP-23 and its ability to form ternary SNARE complexes is required for MC degranulation (Vaidyanathan et al., 2001; Weber et al., 1998). But the exact molecular mechanisms of SNAP-23 function immediately after receiving an activation trigger in a MC are not known except for the concomitant transient phosphorylation of SNAP-23 during regulated exocytosis (Hepp et al., 2005). Our previous studies (Hepp et al., 2005) have shown that SNAP-23 gets phosphorylated at Thr102, Ser95 and Ser120. Among these, Thr102 phosphorylation site remains constitutively phosphorylated and other two are inducible in nature. Further it is known that impairment of induced phosphorylation results in reduced exocytosis (Hepp et al., 2005). In this study we looked at SNAP-23 localization in MCs immediately after receptor cross-linking, and the role of SNAP-23 phosphorylation in this process. One earlier report using permeabilized MCs reconstituted with purified cytosol from rat brain, showed that SNAP-23 relocated from plasma membrane lamellipodia to internal structures within seconds of receiving a
calcium trigger (Guo et al., 1998). Our model system for activation of MCs uses cross-linking of DNP-specific IgE sensitized MCs with the multivalent antigen DNP-BSA mimicking MC activation during an allergen challenge (Gould et al., 2003).

During our microscopic study of SNAP-23, we observed that SNAP-23 membrane association changed dynamically upon allergen trigger and some of it seemed to relocate to intracellular sites. Membrane cytosol fractionation experiments revealed that majority of SNAP-23 was still membrane associated but a significant proportion now resided in the cytosol of MCs. So, a proportion of SNAP-23 translocated to internal organelle membranes and a small portion translocated to the cytosol. Earlier in various cell types t-SNAREs, and SNARE interacting proteins important for exocytosis were shown to recycle between different internal organelles. Syntaxin-3 and Syntaxin-4 were shown to recycle from plasma membrane to TGN and Rab-11 positive recycling endosomes, respectively, in rat kidney cells (Band et al., 2002; Band and Kuismenan, 2005). In dendritic cells Syntaxin-3 is generally expressed in cytosol but upon LPS stimulation it translocates to plasma membrane to mediate IL-6 secretion (Collins et al.). After ionomycin stimulation SCAMP-5 relocates to recycling endosomes from Golgi compartments (where it co-localized with Syntaxin-6) and subsequently to plasma membrane during TNF secretion from activated macrophages and co-localizes with Syntaxin-4/SNAP-23 (Murray and Stow, 2014). Similarly, SNAP-23 relocation in MCs in response to a physiological trigger may be important for mediating some important fusion steps during MC mediator release.

To specifically define the internal locations to which SNAP-23 relocates on MC activation, we took help from our previous studies showing that a large proportion of MC secretory granules are lysosomal in nature (Puri and Roche, 2008). When we co-stained resting MCs with LAMP-3 antibody almost no co-localization between LAMP-3 and SNAP-23 was observed. But, 10 min after receptor cross-linking significant amount of co-localization between SNAP-23 and LAMP-3 was observed, showing relocation of SNAP-23 to LAMP positive internal late endosomal/lysosomal compartments. Similar observations were made in live cell imaging with SNAP-23 and lysotracker as lysosomal marker. We have also shown that LAMP positive secretory granule in rodent MCs harbor cargo like serotonin or cathepsin D and the t-SNARE Syntaxin-3 (Puri et al., 2003; Puri and Roche, 2008). So, we co-stained MC with either serotonin or Syntaxin-3 specific antibodies respectively and found no co-localization of plasma membrane associated SNAP-23 with granule associated serotonin or Syntaxin-3 in resting MCs, but a very significant co-localization of SNAP-23 with serotonin and Syntaxin-3, 10 min after receptor cross-linking. This shows conclusively that SNAP-23 relocates to membrane of internal secretory granules.

The main questions are how and why does SNAP-23 end up on internal membranes in MCs early after receptor cross-linking. Since some SNAP-23 still remained associated with plasma membrane, only a portion of the total pool relocates to internal membranes. There are two possible mechanisms by which SNAP-23 may relocate to internal membranes. One possibility is that it dissociates from plasma membrane, enters cytosol, and again binds to internal lysosomal membranes. In fact, we do see a small but significant increase in SNAP-23 in the cytosolic fraction 10 min after receptor crosslinking. This could be due to SNAP-23 on its way to relocate to internal membranes. There are reports showing that extent of palmitoylation determines the differential membrane association of a protein (Greaves and Chamberlain, 2011). May be the induced phosphorylation of SNAP-23 during exocytosis (Hepp et al., 2005) regulates the palmitoylation of SNAP-23 so that it dissociates from plasma membrane and associates with lysosomal membranes. The other possibility is that immediately after stimulation one or a few granules release their content via “kiss and run” mechanism and quickly SNAP-23 relocates with this granule. It has been reported earlier that MCs exhibit “kiss and run” and cavicapture type of transient granule-plasma membrane fusion to maintain their granularity and to retain the capacity of undergoing repeated exocytosis (Balseiro-Gomez et al., 2016; Cohen et al.). Further, a recent study involving AFM and TEM detailed the capture of a typical porosome on activated RBL mast cells, where membrane bound secretory vesicles dock and fuse (Deng et al., 2010; Jena, 2012). Studies on pancreatic acinar cells, whose secretion is also dependent
on the t-SNARE SNAP-23, have revealed selective presence of SNAP-23 at the base of porosome, the site of secretory vesicle docking and fusion (Jena, 2012). We also could never find any LAMP translocating to plasma membrane after activation, ruling out full fusion between lysosome/granule and plasma membrane. By real time imaging, we have visualized SNAP-23 relocation to internal granule membranes including lysosomes that may be involved in homotypic fusion during compound exocytosis as we have seen SNAP-23 in multiple granule fusion (homotypic fusion) just beneath the plasma membrane, upon activation. Granule-granule homotypic fusion is known to require SNARE rosette formation (Hammel and Meilijson, 2012). Another, least likely possibility is that it is the newly synthesized SNAP-23 which associates with the internal locations, and the older SNAP-23 is still associated with plasma membrane. But, in our experiments, the amount of total SNAP-23 was the same in resting, IgE-sensitized, and receptor cross-linked stages by western blot analysis of endogenous SNAP-23, or flow cytometric analysis of transfected GFP-SNAP-23. So, it is unlikely that in the short time span of 10 min after activation newly synthesized SNAP-23 associates with internal locations in addition to the plasma membrane associated older pool. In other studies involving secretory cells from adrenal medulla, and pancreatic beta cells, t-SNARE SNAP-25 was shown to be important for sequential exocytosis and was supplied to primary granules by lateral diffusion from plasma membrane on stimulation (Kishimoto et al., 2006; Takahashi et al., 2004).

We have shown that SNAP-23 gets differentially phosphorylated (Hepp et al., 2005) in resting and activated MCs. In resting MCs, SNAP-23 is phosphorylated at Thr\textsuperscript{102}, and this basal phosphorylation is not affected by activation. Ser\textsuperscript{95} and Ser\textsuperscript{120} are transiently phosphorylated immediately after activation, and mirror the kinetics of MC secretion. Both, the MC secretion and induced SNAP-23 phosphorylation peak at 5-20 min, and show a decrease thereafter. Since all three major phosphorylation sites are in the linker region of SNAP-23, in close proximity to the conserved cysteine residues which may play an important role in anchoring SNAP-23 to membrane, we decided to study the role of SNAP-23 phosphorylation in its membrane associations in resting or activated MCs, respectively. Transfection, and expression of basal phosphorylation mutant SNAP-23 T102A, which cannot be phosphorylated, revealed that, a large portion of SNAP-23 T102A accumulated in cytosol in resting MCs and the situation remained the same after activation of MCs. Also, SNAP-23 T102A transfected MCs showed a 50% inhibition in exocytosis in comparison to controls. These results indicate that basal phosphorylation of SNAP-23 at Thr\textsuperscript{102} is important for initial membrane association of SNAP-23. Due to the proximity of this site to conserved cysteine in the linker region of SNAP-23, it may function by affecting the palmitoylation of these cysteine residues. Or, it may affect initial association with membrane for palmitoylation, either by facilitating binding to some chaperone (Liu et al., 1996) or by changes in overall hydrophobicity (Polyansky and Zagrovic, 2012). Previous studies have indicated that the first association of similar proteins with membrane may be by some mechanism other than palmitoylation (Dunphy and Linder, 1998). For example, members of the Src family of tyrosine kinases or G protein \(\alpha_{i1}\) subunit are cotranslationally myristoylated, and this helps in rapid membrane association (van't Hof and Resh, 1997) bringing them in close proximity of cellular palmitoyltransferases localized to intracellular membranes (Berthiaume and Resh, 1995; Dunphy et al., 1996; Liu et al., 1996). SNAP-23 is not myristoylated, leaving unresolved the mechanism by which SNAP-23 initially associates with membranes. SNAP-25, the key t-SNARE in neuronal cells that mediates synaptic vesicle release, gets palmitoylated for its membrane association at steady state level in neuroendocrine cells. But its initial plasma membrane association depends on interaction with Syntaxin 1 while still in cytosol (Vogel et al., 2000). Hence some similar mechanism may be facilitated by Thr\textsuperscript{102} phosphorylation in SNAP-23 in MCs for efficient initial association with plasma membrane.

Further, when the inducible phosphorylation sites, Ser\textsuperscript{95}, and Ser\textsuperscript{120}, of SNAP-23 were mutated to S95A/S120A for phospho negative, and S95D/S120D for phospho mimetic mutants respectively, the dynamic regulated changes in SNAP-23 subcellular localization on MC activation were completely compromised. Membrane/cytosol fractionation, and microscopy studies revealed that the phospho negative SNAP-23 S95A/S120A mutant was
able to dissociate from plasma membrane on activation as more of it ended up in cytosol in comparison to control, but unable to associate with internal LAMP-3 positive membranes. Hence, transient induced phosphorylation of SNAP-23 at Ser<sup>95</sup> and Ser<sup>120</sup> seems to be important for association of SNAP-23 with internal lysosomal membranes. This conclusion was further validated by subcellular localization studies on the transfected phospho mimetic SNAP-23 S95D/S120D mutant in resting and activated MCs. In both stages, the SNAP23 S95D/S120D mutant shows a higher co-localization with LAMP-3 containing internal membranes. Previously, in other studies, phosphorylation of claudin 1 (French et al., 2009), and beta-catenin (Qian et al., 2014) has been shown to regulate their subcellular localization and functions. Likewise, we have found that phosphorylation regulates membrane relocation of SNAP-23 during MC exocytosis. That means induced phosphorylations are mediating the relocation and internal membrane association of SNAP-23 to mediate the granule-granule fusion during MC mediator release. The present study reveals, for the first time, that, induced phosphorylation of SNAP-23, has a role in dynamic changes in subcellular localizations of SNAP-23 in MCs undergoing degranulation. Mutations in phospo sites lead to a partial inhibition in exocytosis. So, maybe the induced phosphorylation of SNAP-23 is involved in bringing SNAP-23 to the right location, enabling it to participate in granule-granule fusion, which is an important step in compound exocytosis. The phosphomimetic mutant of SNAP-23 shows good association with internal LAMP positive membranes, but its overall association with membranes is significantly lower than that of SNAP-23 wildtype. So, more than 50% of this mutant remains displaced to cytosol, and hence fails to reach the right locations. These results indicate that the transient nature of phosphorylation is very important. The dephosphorylation following the phosphorylation may be important for recycling of SNAREs by priming or recycling of SNAP-23 back to plasma membrane (Hepp et al., 2005; Puri et al., 2003). As the SNAP23 S95D/S120D mutant is unable to show this dephosphorylated stage it is either stuck on internal membranes or in the cytosol, and hence causes a significant inhibition in exocytosis.

On the basis of our findings we present a model for the molecular mechanisms of MC degranulation regulated by SNAP-23 phosphorylation (Fig. 7). Based on our results we hypothesize a dynamic pathway of SNAP-23 functioning that is represented by a schematic diagram (Fig. 7). Our earlier studies indicated localization of Syntaxin-3 and VAMP-7/8 on LAMP-3 positive granules in MCs (Puri et al., 2003; Puri and Roche, 2008). So, SNAP-23 could be the t-SNARE counterpart in the ternary complex (where it is still present in phosphorylated state) for the SG-SG fusion to occur. During physiological trigger SNAP-23 relocation may be required for homotypic fusion of SGs followed by compound exocytosis in MCs. Till date there are no clear reports regarding molecules that are associated in the ternary SNARE complexes that mediate homotypic fusion in MCs. To our knowledge this is the first report showing SNAP-23 relocation to internal membranes in activated MCs during exocytosis under physiological conditions, and a role for SNAP-23 basal and induced phosphorylations in its dynamic membrane associations. Though detailed molecular mechanisms remain to be determined, we have clearly shown the dynamic nature of SNAP-23 membrane association, may be to regulate different fusion steps in activated MCs leading to discharge of various inflammatory mediators.
Materials and Methods

Cell Culture

Rat basophilic leukemia mast cells [(RBL-2H3) were a kind gift from Dr. Paul A Roche (NIH, Bethesda, MD, USA)], were maintained in equal parts minimal essential medium (Sigma, MO, USA) and Iscove’s medium (Gibco, life technologies, Grand Island, NY, USA) containing 20% FBS (Gibco, life technologies, Grand Island, NY, USA), 25 mM HEPES (Sigma, MO, USA), and 120 μg ml⁻¹ gentamicin (RBL complete medium) as described in (Hepp et al., 2005). Cells were maintained as sub-confluent monolayers at 37°C in a humidified atmosphere containing 5% CO₂ and passaged with trypsin.

Plasmids

pCMV-FLAG-Rat SNAP-23 wildtype, pCMV-FLAG-Rat SNAP-23 T102A, pCMV-FLAG-Rat SNAP-23 95A/120A, pCMV-FLAG-Rat SNAP-23 95D/120D were a kind gift from Dr. Paul A Roche (NIH, Bethesda, MD, USA). The cDNA encoding full length wild type SNAP-23 and its phosphomutants were subcloned from pCMV-FLAG-Rat SNAP-23 wildtype into EGFP-C2 plasmid (#6083-1) (Clontech, CA, USA) by using EcoRI & ApaI restriction sites to generate amino terminal GFP-tagged protein (called GFP-SNAP-23). The integrity of subcloned plasmids was confirmed by sequencing from GCC Biotech and SciGenom Labs Pvt Ltd., India.

Antibodies

Polyclonal rabbit anti-serum recognizing the SNAP-23 carboxyl terminus was a gift from Paul A Roche (National Institutes of Health, Bethesda, MD, USA). Anti–DNP IgE (clone TIB 142) was obtained from the American Type Culture Collection (Manassas, VA, USA). Mouse anti-CD63/ LAMP 3 monoclonal antibody (mAb) AD1 (# 551458,1:100), Mouse Anti-Rat trans-Golgi network (TGN) 38 antibody (# 610899,1:100) and Mouse Anti-Golgi matrix protein (GM) 130 antibody (# 610822, 1:100) (BD Biosciences, San Diego, CA, USA), and anti-GFP rabbit mAb from Clontech (CA, USA) were used in this study. Mouse anti Serotonin antibody (# M0758, 1:50) was from Dako (Carpinteria, CA, USA). Alexa dye-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR, USA) [anti Rb Alexa 546: A11035; anti mouse Alexa 546: A11030; anti mouse Alexa 488: A11001 dilution 1:500]. Lysotracker Red (# L7528) was from Molecular Probes (Eugene, OR, USA). Anti-Rabbit Protein A-HRP conjugated antibody (# 7300-05, 1:7000, Southern Biotech, Birmingham, AL, USA) and anti-mouse IgG-HRP conjugated antibody (# 1031-05, 1:7000, Southern Biotech, Birmingham, AL, USA) were used. Antibody recognizing Syntaxin-3 (# ab133750, 1:800) (rabbit monoclonal) was obtained from Abcam (UK).

Transfection of RBL cells

Transfection of RBL cells was performed as described earlier (Puri et al., 2003) with some modifications. Briefly, exponentially growing RBL cells (10x10^6/ 0.5 ml serum free RBL media) were transfected by electroporation (320 mV, 950 μF) with 20 μg DNA. Immediately after electroporation, the cells were plated in RBL complete medium and analyzed 24 h later. For microscopy, transfected cells were plated on coverslips and then treated as per requirement.

Stimulation of RBL cell exocytosis in transfected RBL cells

RBL cells were transfected by electroporation with human growth hormone (2 ug) expression vector together with empty GFP vector or GFP-SNAP-23 wildtype or phospho mutant (20 ug). After 4-5 h culture, the cells were sensitized with IgE and after 16-18 h they were mock-stimulated or stimulated with DNP-BSA as described before (Hepp et al., 2005). The amount of human 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 (Puri et al., 2003). 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.
Confocal microscopy

RBL cells with or without transfection were seeded on 10 mm diameter coverslips. For indirect immunofluorescence analysis, the cells were either fixed with 4% paraformaldehyde (PFA) in PBS for 30 min and excess paraformaldehyde quenched with 50 mM NH4Cl in PBS or with cold methanol at -20°C for 4 min. After washing, the fixed cells were permeabilized with 1% IGEPAL (Sigma, MO, USA) in the presence of 3% normal goat serum (Sigma, MO, USA) and 0.05% saponin (SD Fine Chem. Limited, Boisar, India) in PBS. The cells were then incubated with 3% normal goat serum and 0.05% saponin in PBS for 1 h at RT to prevent nonspecific protein binding. Primary Abs diluted in the same buffer were added to the cells, and incubation was conducted for 1 h at RT. After washing, the cells were incubated for 30 min in the presence of secondary goat Abs conjugated to Alexa Fluor 546 (red) (Molecular Probes, Eugene, OR, USA). As a control, samples were stained with an irrelevant antibody and no staining was observed in the respective channel for all confocal fluorescence microscopy experiments. Confocal images were collected with Olympus Fluoview FV1000 microscope at 100X magnification (sometimes with 2X zoom), an optical slice thickness of 1.0 μm. Image Z-stacks were collected through the depth of the cell using 0.4μm step size. Co-localization analysis was done for each plane of the individual image stacks using the co-localization analysis feature of the Fluoview software Ver.1.7a (Olympus). Briefly, individual channels were thresholded to include the structures of interest; regions of interest were then drawn to encompass the structures, resulting in scatter plots being generated and co-localization coefficients calculated. The colocalization coefficients represent co-localization in the green channel with respect to the red channel. Single images were exported from the Fluoview software Version 1.7a Software and organized into figures using Microsoft PowerPoint 2007.

Real time Imaging of GFP SNAP-23 expressing cells

RBL cells were transfected with EGFP-SNAP-23 plasmid, plated in a 3.5 cm culture dish and sensitized with anti-DNP IgE in RBL complete medium overnight at 37°C. Next day these were incubated with Lysotracker red [a dye which stains the lysosomes and secretory granules as well in live cells (Marchini-Alves et al.)] for 2 h in RBL complete medium. After washing with phenol red free RPMI medium they were either mock stimulated or stimulated with 100 ng/ml DNP-BSA and observed by a live cell imager Andor Spinning Disk Confocal microscope (Nikon Eclipse TiE, Software-Andor iQ 2.7) in 5% CO2 chamber at 37°C. The movies were captured 2 min after the addition of allergen and continued for 10 min. At least 5 movies were captured in the above manner for each separate experiment. All the images were analyzed by NIS element AR ver4.

Membrane-Cytosol fractionation

Membrane-cytosol fractionation was done as described earlier (Hepp et al., 2005). Briefly, transfected RBL mast cells were harvested after 24 h and resuspended in hypotonic buffer (10 mM Tris, 1 mM KCl, 1 mM EGTA, 0.5 mM MgCl2, pH 7.4) containing protease inhibitors (5 mM iodoacetamide, 50 mM PMSF, and 0.1 mM TLCK) and phosphatase inhibitors (5 mM EDTA, 5 mM EGTA, 50 mM NaF, 10 mM Na4P2O7, and 1 mM Na3VO4). They were then disrupted by repeated passage of cells through a 30.5 gauge syringe. Nuclei and unbroken cells were removed by centrifugation at 1000xg and the post-nuclear supernatant was subjected to centrifugation at 100,000xg for 1 h at 4°C to isolate membrane (pellet) and cytosol (supernatant). 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.
**SDS-polyacrylamide gel electrophoresis and Immunoblotting**

RBL cell lysates and membrane-cytosol fractions were boiled in β-mercaptoethanol containing sample buffer and proteins were separated in 12.5% SDS-polyacrylamide gel. Immunoblotting was performed with polyclonal SNAP-23 C-terminus antibody or GFP antibody as previously described (Puri et al., 2003). As secondary antibodies anti-Rabbit Protein A-HRP and anti-mouse IgG-HRP were used. For Immunoblotting separated proteins were transferred to 0.2 μ PVDF membrane (Bio-Rad, USA) and visualized by ECL using Immobilon Western Chemiluminescence HRP substrate (Millipore, MA, USA). Band intensity was determined by Spot Denso (AlphaEaseFC software, Alpha Innotech). 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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**Competing interests**

The authors declare no competing interest.

**Author contributions**

Conceived and designed the experiments: NP. Performed the experiments: PN. Analyzed the data: NP, PN, Contributed reagents/materials/analysis tools: NP. Wrote the paper: NP, PN.

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Fig. 1: Membrane localization of SNAP-23 in resting and IgE cross-linked RBL MCs. (A-B) Representative confocal images showing cross-sections of GFP-SNAP-23 (green) transfected RBL MCs along with DIC images. (A) In resting RBL MCs GFP-SNAP-23 wild type (WT) is associated with plasma membrane (n=28). (B) In receptor cross linked RBL MCs GFP-SNAP-23 WT is also seen in internal locations in cytosol (n=43). The white
arrow heads are indicating GFP-SNAP-23WT in spherical-granule like structures in cytosol. Scale bar=10 µm. (C) Immunoblot by SNAP-23 specific antibody, showing the membrane and cytosol association of endogenous SNAP-23 and transfected GFP-SNAP-23WT. (D) Quantitative analysis of the immunoblots in (C) showing a significant decrease in membrane association of SNAP-23 (both endogenous and transfected SNAP-23 WT) in receptor cross linked. Each data point is a mean ± SEM of at least three independent experiments (*, p ≤ 0.05). (E-F) Representative still images from live cell imaging of GFP-SNAP-23WT transfected RBL MCs with Lysotracker red staining to track lysosomal compartments. SNAP-23 localization at plasma membrane is shown during mock stimulation at three representative time points with no co localization with lysotracker red (E) (derived from live cell). The “Receptor cross linked”-panel (F) is showing the snap shots from a representative video (at least 18 videos were captured), depicting SNAP-23 internal membrane localization. White arrows indicate SNAP-23 association with lysosome and arrow heads mark SNAP-23 associated lysotracker. The scale bar represents 10 µm.
Fig. 2: SNAP-23 shows significant co-localization with lysosomal granule marker LAMP-3 but not with TGN38 and GM130 in allergen stimulated RBL MCs. (A-B) The representative confocal images are showing that endogenous SNAP-23 (green, SNAP-23 Ab) does not relocate to Trans Golgi network or Golgi membrane (red, TGN-38 Ab (n=71, IgE sensitized; n=100, receptor cross linked) and GM-130 Ab (n=100, IgE sensitized; n=70, receptor cross linked) after receptor cross linking of MCs. (C) Representative confocal image is showing the GFP-SNAP-23 WT (green) co-localization with lysosomal granules (red, granule marker LAMP-3-specific Ab). A high amount of co-localization (yellow, overlay) of GFP-SNAP-23 WT with LAMP-3 containing granules was observed after receptor cross linking of MCs (n=15). In all resting states SNAP-23 resides on plasma membrane.
with no co-localization with any internal organelle marker. Scale bar represents 10 μm. (D) The graph representing the co-localization of SNAP-23 (both endogenous and transfected) with the counterstained markers (above) in terms of Pearson Coefficient. Each data point is a mean ± SEM of three independent experiments (**, p ≤ 0.005).
Fig. 3: SNAP-23 shows co-localization with secretory lysosomes in allergen activated RBL MCs. (A) GFP-SNAP-23 WT (green) relocates to internal serotonin (red, anti-Serotonin Ab) containing granules after MCs receptor cross linking (n=20). The inset image shows an enlarged view of over-lapping region indicated by the white box and the white arrows depicting green-SNAP-23 associated granules containing red serotonin cargo within them. (B) In IgE-sensitized MCs GFP-SNAP-23 (green) does not co-localize with STX-3 containing granules (red, STX-3 specific antibody) (n=32). But after the allergen cross linking a significantly high amount of SNAP-23 was found to co-localize with STX-3 containing secretory granules (n=42). Scale bar represents 10 μm. (C) The graph representing the co-localization of GFP-SNAP-23WT with the counterstained markers (above) in terms of Pearson Coefficient. Each data point is a mean ± SEM of at least three independent experiments (*, p ≤ 0.05; **, p ≤ 0.005).
**Fig. 4: SNAP-23 phospho mutants and their dynamic membrane association:** (A) Sequence alignment showing the SNAP-23WT and other phospho mutants (T102A, S95A/S120A, S95D/S120D) in the linker region. Protein sequences obtained from their respective DNA sequences by using “DNA to Protein translation tool” (http://insilico.ehu.es/translate/index.php). (B) Immunoblots showing the membrane and cytosol association of GFP-SNAP-23 phospho mutants (by SNAP-23 specific antibody). Lanes are cut from the same blots and repositioned (for GFP-SNAP-23 T102A mutant). (C) The band intensities were quantified by spot denso Alpha EaseFC software. The percent membrane association of SNAP-23 reveals that the mutation of the above residues affects SNAP-23 dynamic association as well as cytosolic accumulation. Each data point is a mean ± SEM of three independent experiments (*, p ≤ 0.05; **, p ≤ 0.005; ***, p ≤ 0.0005 NS, not significant).
Fig. 5: Effects of mutations at $T^{102}$, $S^{95}$ and $S^{120}$ in membrane association of SNAP-23: (A-C) Representative confocal microscopy images are showing the GFP-SNAP-23 $T^{102}$A ($n=17$) and GFP-SNAP-23 $S^{95A/S120A}$ ($n=34$) (green) do not co-localize with LAMP-3 positive granules (anti-LAMP-3 Ab, Red) in IgE-sensitized and receptor cross linked MCs. But GFP-SNAP-23 $S^{95D/S120D}$ shows high level of association with LAMP-3 ($n=20$) in IgE sensitized and receptor cross linked MCs as well. Scale bar represents 10 μm.
Fig. 6: Expression of phospho mutants affects exocytosis from transiently transfected RBL MCs: The bar graph shows the extent of exocytosis as net (percent of total) release of hGH secretion reporter from transfected RBL MCs on receptor cross linking. Transfection with SNAP-23 T102A, SNAP-23 S95A/S120A and SNAP-23 S95D/S120D causes a significant inhibition in net hGH release from transfected RBL MCs in comparison to cells with WT SNAP-23. The data shown are mean ± SEM of three independent experiments, and asterisks indicate statistically significant differences in hGH release between SNAP-23 WT vs mutant transfected RBL MCs (*, p ≤ 0.05; ***, p ≤ 0.0005).
Fig. 7: Schematic representation of relocation of SNAP-23 during regulated exocytosis of MCs. (A) In resting or IgE sensitized MCs, SNAP-23 is synthesized in cytosol and phosphorylated at Thr\textsuperscript{102} which is important for its initial plasma membrane association. (B) After 10 min of the cross linking of IgE with DNAP-BSA, SNAP-23 relocates from plasma membrane to internal organelle membrane by two possible mechanisms (1, 2 / 2a) and gets phosphorylated at Ser\textsuperscript{95} and Ser\textsuperscript{120} positions. This relocated SNAP-23 may mediate granule-granule fusion (3) and subsequently compound exocytosis from MCs (4).
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<th>Transfection</th>
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<td>0.62±0.06&lt;sup&gt;NS, NS&lt;/sup&gt;</td>
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Shown above is the extent of colocalization of SNAP-23 mutants with LAMP-3 in comparison to SNAP-23WT in IgE sensitized and activated mast cells. In the crosslinked stage the statistics show first the comparison between SNAP-23 wildtype and the respective phospho mutant and then the comparison between IgE sensitized and crosslinked stages for each of the specific mutant.
Fig. S1: Mast cell activation by IgE cross-linking. (A) The graph is showing the kinetics and cumulative secretion of β-hexosaminidase from receptor cross linked RBL MCs at indicated time points. (B) Immunoblot with a Rabbit anti-phospho-SNAP-23-Ser120 antiserum is showing appearance of a band corresponding to Ser120P-SNAP-23 5 min after receptor cross linking and its disappearance at 30 min, while the total amount of SNAP-23 remains constant. (C) The graph is derived from the quantitative analysis of the immunoblot in (B), representing the phosphorylation kinetics of SNAP-23 after receptor cross linking. (D) Immunoblot (by SNAP-23 Ab) showing the total amount of SNAP-23 protein (endogenous/ GFP-SNAP-23WT) is constant in resting, IgE sensitized and receptor cross linked MCs. (E) The table represents the Mean Fluorescence Intensity (MFI) values of flow cytometric analysis of GFP-SNAP-23WT transfected RBL cells in the respective conditions. It shows that the fluorescence intensity of GFP-SNAP-23 does not change during MCs activation. Statistical calculations were made using a one-tailed distribution in a two-sample equal variance Student’s t test. Each data point is a mean ± SEM of at least three independent experiments (*, p≤ 0.05).
Fig. S2: Expression of SNAP-23 phospho mutants in RBL MCs. (A) Equivalent amounts of cell lysate from RBL cells expressing GFP tagged SNAP-23WT, SNAP-23T102A, SNAP-23 S95A/S120A, or SNAP-23 S95D/S120D were analysed by immunoblot analysis using a SNAP-23 specific antibody. This antibody detects endogenous SNAP-23 and GFP SNAP-23 equally well. (B) The band intensities were quantified by spot denso Alpha EaseFC software and percent expression of transfected SNAP-23 with respect to endogenous was determined. The bar graph shows almost equal level of expression in case of SNAP-23WT, SNAP-23 S95A/S120A, and SNAP-23 S95D/S120D mutant but significantly lower expression of SNAP-23T102A mutant. Each data point is a mean ± SEM of three independent experiments (**, p≤ 0.005).
SNAP-23 movements were captured during mock stimulation (A) and allergen stimulation (B). Movies were captured starting at 2 min after the addition of medium alone or allergen, DNP-BSA. Total 300 frames were taken. Display rate is 14 frames per second. (A) Shows that during mock stimulation SNAP-23 remains associated with plasma membrane and there is not much movements of lysotracker towards plasma membrane. (B) Representative movie (of 18 captured movies) shows, upon receptor cross linking of RBL mast cells showing that SNAP-23 relocates to internal granule membranes that are undergoing homotypic fusion and compound exocytosis. Red Lysotracker containing granules surrounded by green GFP-SNAP-23WT can also be visualized during the course of acquisition.

Supplementary Movies 1. Partial relocation of SNAP-23 to internal membranes during mast cell exocytosis. IgE sensitized transfected RBL cells expressing GFP SNAP-23 were stained with Lysotracker. Real time imaging of SNAP-23 movements were captured during mock stimulation (A) and allergen stimulation (B). Movies were captured starting at 2 min after the addition of medium alone or allergen, DNP-BSA. Total 300 frames were taken. Display rate is 14 frames per second. (A) Shows that during mock stimulation SNAP-23 remains associated with plasma membrane and there is not much movements of lysotracker towards plasma membrane. (B) Representative movie (of 18 captured movies) shows, upon receptor cross linking of RBL mast cells showing that SNAP-23 relocates to internal granule membranes that are undergoing homotypic fusion and compound exocytosis. Red Lysotracker containing granules surrounded by green GFP-SNAP-23WT can also be visualized during the course of acquisition.