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Characterization of Molecular Interactions between Proteins of the Neurotransmitter Release Site.

an investigation of intramolecular interactions of the N-terminal region of Munc13-1

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SUMMARY

Interneuronal communication is mediated by neurotransmitters, which are stored in presynaptic vesicles during the resting state. An intracellular calcium influx through voltage-gated calcium channels triggers exocytosis in response to action potentials. Neurotransmitter release is a rapid, multistep process, spatially restricted to the active zones of the presynaptic nerve terminal. Synaptic vesicles dock and fuse with the presynaptic plasma membrane, before releasing neurotransmitters to the synaptic cleft, which are then received by receptors at the postsynaptic plasma membrane. A complex protein machinery including five large multidomain proteins is implicated in regulation of a series of events leading up to neurotransmitter exocytosis. These proteins: Aczonin, Bassoon, RIM, Munc13 and Cast, have been identified in previous experiments as constituents of active zones. A recombinant protein construct of the N-terminal region of Munc13-1 (Munc13-1-NT), which encompasses Mu1 and Mu2, has particularly been shown to interact with these active zone proteins *in vitro.*

My experimental design was twofold: first I made recombinant constructs representing various lengths of the N-terminal domain of Munc13-1, and then used GST-pulldown assays to investigate protein-protein interactions between Munc13-1-NT and the other active zone proteins. Similar results indicating an interaction between Mu1 and Mu2 were obtained from the SDS-PAGE and Western blots.

In this study, I demonstrate an intramolecular interaction of two distant domains of Munc13-1-NT that is unaffected by introduction of phosphomimetic mutations (T117E or S385E), but is abolished by calmodulin. This observation is especially exciting because it implicates calmodulin, a protein previously known to mediate synaptic plasticity, in the regulation of the Mu1-Mu2 intramolecular interaction. Given that some neurological diseases (like Parkinson’s and Huntington's disease), are due to imbalances of neurotransmitters, understanding the precise regulation of neurotransmitter exocytosis is a critical step in devising strategies against these neurological disorders.

Glossary

Active zones are unique sites at the presynaptic plasma membrane, where synaptic vesicles are targeted, clustered and docked, before they fuse with the presynaptic plasma membrane to release neurotransmitters (5,6,9,13-18, 20-25).

Calcium is the most plentiful mineral element in the human body, where it is in form of calcium ions, with essential roles in cell signaling, muscle contraction, nerve signaling, fertilization and cell division (13).

The *CALcium MODULated proteIN (calmodulin)* is a highly conserved protein through evolution, that is abundant in the cytoplasm of all higher cells. It acts as an intermediary protein that senses calcium levels and relays signals to various calcium-sensitive enzymes, ion channels and other proteins (13).

Chemical synapses are specialized sites of information exchange between neurons and their target cells. Synapses are usually between the axon of the sending cell and a dendrite (or membrane of an effector cell) of the receiving cell (4,13).

Exocytosis is a cellular process whereby intracellular vesicles in the cytoplasm fuse with the plasma membrane and release or "secrete" their contents into the extracellular space (13).

Neurons are core components of the brain and spinal cord in vertebrates and the ventral nerve cord in invertebrates. They are electrically excitable cells in the nervous system that process and transmit information. It is estimated that the human brain constitutes about 10^{11} neurons and 10^{14} synapses (13).

Neurotransmitters are molecules used to relay, amplify and modulate signals between a neuron and it's target cell. Each neurotransmitter sends a specific message to the receptor cell. Neurotransmitters may be in form of amino acids, peptides and monoamines. Glutamic acid and gamma aminobutyric acid (GABA) are the major neurotransmitters (13).

Phosphomimetic mutations are amino acid substitutions that are predicted to mimic a state of constitutive phosphorylation. In this study I introduced two phosphomimetic mutations: T117E (substitution of a threonine 117 for a glutamate) and S385E (substitution of serine 385 for glutamate.) Phosphomimetic mutations are used to analyze the role of phosphorylation in protein-protein interactions (9).

Synaptic plasticity refers to the ability of synapses to be changed and moulded in response to a given neurotransmitter. This property allows for varied interneuronal communication, which is critical in learning and forming memories (7).

Glutathione S-transferase (GST) pull-down assay is an extensively used method to identify protein-protein interactions, confirm known interactions and to map interaction sites. In this assay, a GST fusion protein is immobilized on glutathione sepharose beads that serve as a solid phase, then the mixture is incubated with a cell lysate or purified protein. Nonbound material is washed off the column, and subsequently the binding complex is eluted.

Upon elution, the mixture is resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by coomassie staining, silver staining, or Western blot (2).

Glutathione-S-transferase (GST) tags are epitopes commonly used for expression and purification of GST-fusion proteins. The glutathione resin used in GST-fusion purification system, selectively binds the GST-tagged protein under normal conditions, allowing the specific protein of interest to be separated from whole cell extracts rapidly and efficiently (Clontech).

Polyhistidine (His) tags are often used for affinity purification of His-tagged recombinant proteins that are expressed in *Escherichia coli.* The Nickel Nitrilotriacetic acid (Ni-NTA)-agarose used in His purification system is composed of Ni-NTA coupled to Sepharose CL-6B. It contains a nickel ion to which the polyhistidine tag binds with micromolar affinity. As such, a specific His- tagged protein may be separated from other cellular proteins. Increasing concentrations of imidazole (100-250 mM) are used to dissociate His-tagged proteins from the Ni-NTA resin (Qiagen).

Mu1 and Mu2 refer to individual parts of the Munc13-1 N-terminus corresponding to amino acids 1-320 and amino acids 363-651, respectively.

INTRODUCTION

The nervous system

Multicellular animals are particularly dependent on a robust nervous system in order to maintain, adjust or respond to their internal and external environments. Almost every organ is under the control of the nervous system, which relies on a series of positive and negative feedback loops to provide regulation. The nervous system is subdivided into a central nervous system (CNS) which includes the brain and spinal cord, and the peripheral nervous system (PNS) that connects the CNS to other parts of the body, and is composed of nerves (bundles of neurons) (14). Understanding neurotransmitter release is especially relevant because some neurological diseases, for example Parkinson's disease and Huntington's disease, are due to imbalances of neurotransmitters. Parkinson's is due to a dopamine deficiency, whereas Huntington's disease is attributed to malfunctioning of an inhibitory neurotransmitter (11). Dopamine is a hormone and neurotransmitter that is found in both vertebrates and invertebrates and activates five types of dopamine receptors (D1-D5) (13). The bacterium *Clostridium tetani* produces a toxin that prevents the release of gamma amino butyric acid (GABA). GABA is important in the control of skeletal muscles and therefore inhibition of its release is potentially fatal, when it effects the muscles used in breathing (13).

Interneuronal communication

Communication between neurons and their target cells, which include other neurons, muscle or gland cells, is mediated by neurotransmitters (2,3,8,15,18,22). Synaptic junctions constitute a presynaptic terminal, synaptic cleft and postsynaptic terminal, arranged asymmetrically. Chemical synapses release neurotransmitters from synaptic vesicles at active zones of the presynaptic terminal to the synaptic cleft, where they diffuse and activate receptors at the postsynaptic plasma membrane. Typically, presynaptic action potentials trigger intracellular calcium influx via voltage gated calcium channels, thereby activating neurotransmitter exocytosis. Neurotransmitter release is spatially restricted to unique sites (active zones) along the presynaptic terminal (5,6,9,13-18, 20-25).

Maturation of Synaptic vesicles

Prior to neurotransmitter release, a few synaptic vesicles close to the active zone mature into a fusion-competent, primed state and are triggered by intracellular calcium levels to fuse with

the plasma membrane. Then, neurotransmitters are taken up by synaptic vesicles and the vesicles are freed from cytoskeletal elements, targeted, clustered and docked at the active zone. During docking, the vesicle and presynaptic membrane line up in a fusion-ready state, before the membranes fuse to create a fusion pore which collapses into the presynaptic membrane and exocytosis occurs.

After exocytosis, synaptic vesicles are endocytosed and recycled. Synaptotagmin is a calcium sensor that is implicated in initiating this vesicle fusion (3,12). Synaptic plasticity is attributed to the fluctuations in readily releasable pool of vesicles. Short term potentiation (millisec-sec) accounts for sensory adaptation and rhythm generation, whereas complex brain processes, such as learning and memory, are due to long term potentiation (10,23).

Active zone proteins

Neurotransmitter release is an extremely rapid and precise multi step process that is stringently and subtly regulated by a complex protein machinery. This machinery includes five large multi-domain proteins (Munc13, Aczonin, Bassoon, RIM and Cast), enriched at the active zone. It is postulated that these proteins have distinct but interrelated roles and interact with specific partners in a given sequence of events (2-4,7,8,12,15,17).

Pull-down assay

The pull-down assay is an *in vitro* method used to determine physical interaction between two or more proteins. It may be used as an initial screening to assay for previously unknown protein-protein interactions or as a complementary method to confirm the existence of interactions predicted by other techniques such as co-immunoprecipitation, yeast two-hybrid or density gradient centrifugation. In a pull-down assay, an immobile protein (GST-fusion construct) is incubated with a mobile partner (His tagged construct), after which proteins are eluted with SDS loading buffer and analyzed on SDS-PAGE. GST and His tags are useful in the purification step as a tool to discriminate between a protein of interest from other cellular proteins.

Aim of study

The N-terminal domain of Munc13-1 has previously been shown to have binding sites for the other active zone enriched proteins: Aczonin, Bassoon, Cast, and Rim (1-3,6,7,10,12,14). In this study I investigated in detail molecular interactions involving the N-terminal domain of

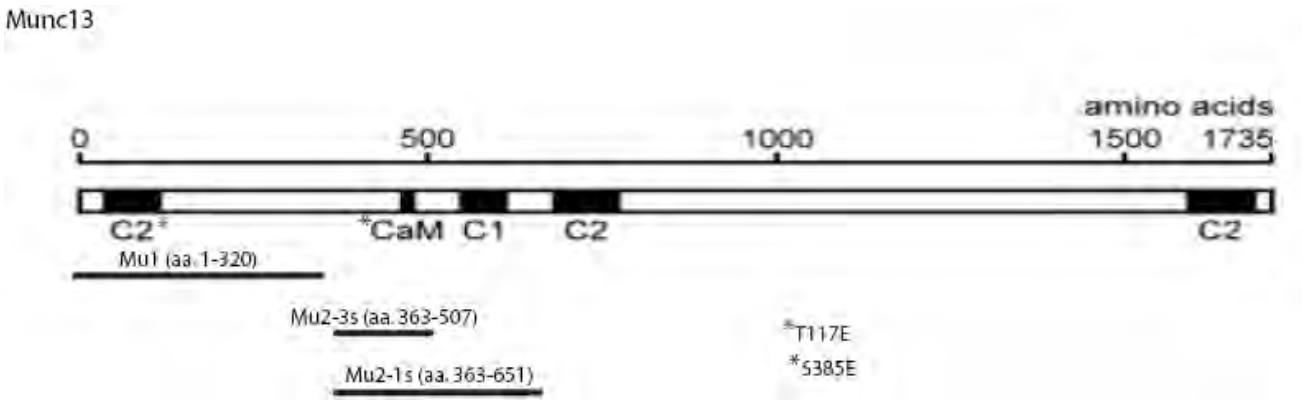
Munc13, particularly Mu1-Mu2 interactions. The role of phosphomimetic mutations and calmodulin on these interactions was also examined.

RESULTS

Recombinant Munc13-1-NT constructs

In the present study I generated various constructs corresponding to different regions of the Munc-13-1 N-terminus (NT) (Fig. 1). Constructs of particular interest were: Mu1, Mu2-1s and Mu2-3s, which were cloned in both pQE-32 and pGEX-4T2 expression vectors for His and GST tagged constructs, respectively. Mu1 (a.a. 1-320) has a C2 domain, Mu2-3s (a.a. 363-507) includes a CaM domain, whereas Mu2-1s (a.a. 363-651) encompasses CaM and C1 domains (Fig 1A). C2 domains are protein structural domains involved in targeting proteins to cell membranes, whereas CaM domains bind calmodulin, as suggested by the name. C1 domains are known to bind an important secondary messenger diacylglycerol (DAG), as well as the analogous phorbol esters (1,19). These constructs represent different domains of Munc13-1-NT and are therefore suitable for investigations of intramolecular interactions *in vitro*. I also introduced a phosphomimetic mutation (S385E) and W460R mutation on the calmodulin site (CaM), to Mu2-1s and Mu2-3s. The phosphomimetic mutation was for studies on the role of phosphorylation, while the W460R mutation was used to analyse the effect of calmodulin on Munc-13-1-NT intramolecular interactions. The dimerization (K32E), and dimerization, phosphomimetic (K32E,T117E), mutant Mu1 constructs were previously generated. Munc13-1-NT constructs used in this study are shown in Tables 6 and 7 (Materials and Methods).

A



B

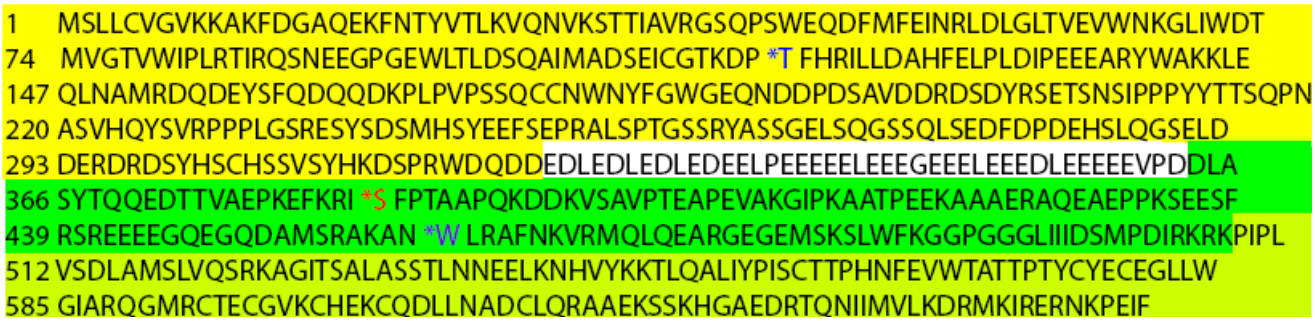


Fig. 1. (A) Schematic overview of Munc13 sequence regions (Kilimann MW, unpublished). Mu1 (a.a. 1-320), Mu2-3s (a.a. 363-507) and Mu2-1s (a.a. 363-651) regions are based on mouse sequence, accession number: NM_021468.) Phosphorylation sites (P)T117E (*blue) and (P)S385E (*black), as well as C2, CaM and C1 domains are shown. **(B)** Amino acid sequence of Munc13 (a.a. 1-651). Mu1 (a.a. 1-320) is highlighted in yellow, Mu2-3s (a.a. 363-507) in green and Mu2-1s (a.a. 363-651) which encompasses green and light green regions, are shown. Phosphorylation sites (P)T117E (blue) and (P)S385E (red), as well as the calmodulin binding site (CaM) W460R (purple) are also indicated. Asterisks (*) denote introduced mutations.

Mu1pETcHis binds to immobilized Mu2 constructs

Previous experiments have shown the N-terminal domain of Munc13 to bind other active zone proteins (1-3,6,7,10,12,14). I analyzed interactions between immobilized GST-Mu2 constructs: Munc13-1-NT; Mu2-1s (a.a. 363-651), the shorter variant Mu2-3s (a.a. 363-507) and Mu1pETcHis (K32E), a dimerization mutant (a.a. 1-320), as well as the dimerization and phosphomimetic mutant (Mu1pETcHis_K32E,T117E) using GST pull down assays and immunoblotting. Immobile GST-Mu2-1s and GST-Mu2-3s were individually incubated with both mobile Mu1 constructs (Fig. 2A). GST-Mu1 (a.a. 2-320) and recombinant GST were also included as positive and negative controls, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that both Mu1 dimerization mutants (48 kDa) were precipitated by the GST-Mu2 constructs, but not positive (GST-Mu1) or negative (GST) controls (Fig. 2A). Anti-cHis antibody which detects the C-terminal His tag on Mu1pETcHis, was further used to confirm these interactions (Fig. 2C). These pull down assays and immunoblots indicated that GST-Mu2 variants (GST-Mu2-1s and GST-Mu2-3s) interacted with mobile Mu1. The Mu2 moiety of the GST-fusion protein bound Mu1pETcHis, since GST alone did not interact with the mobile partner (Fig. 2B).

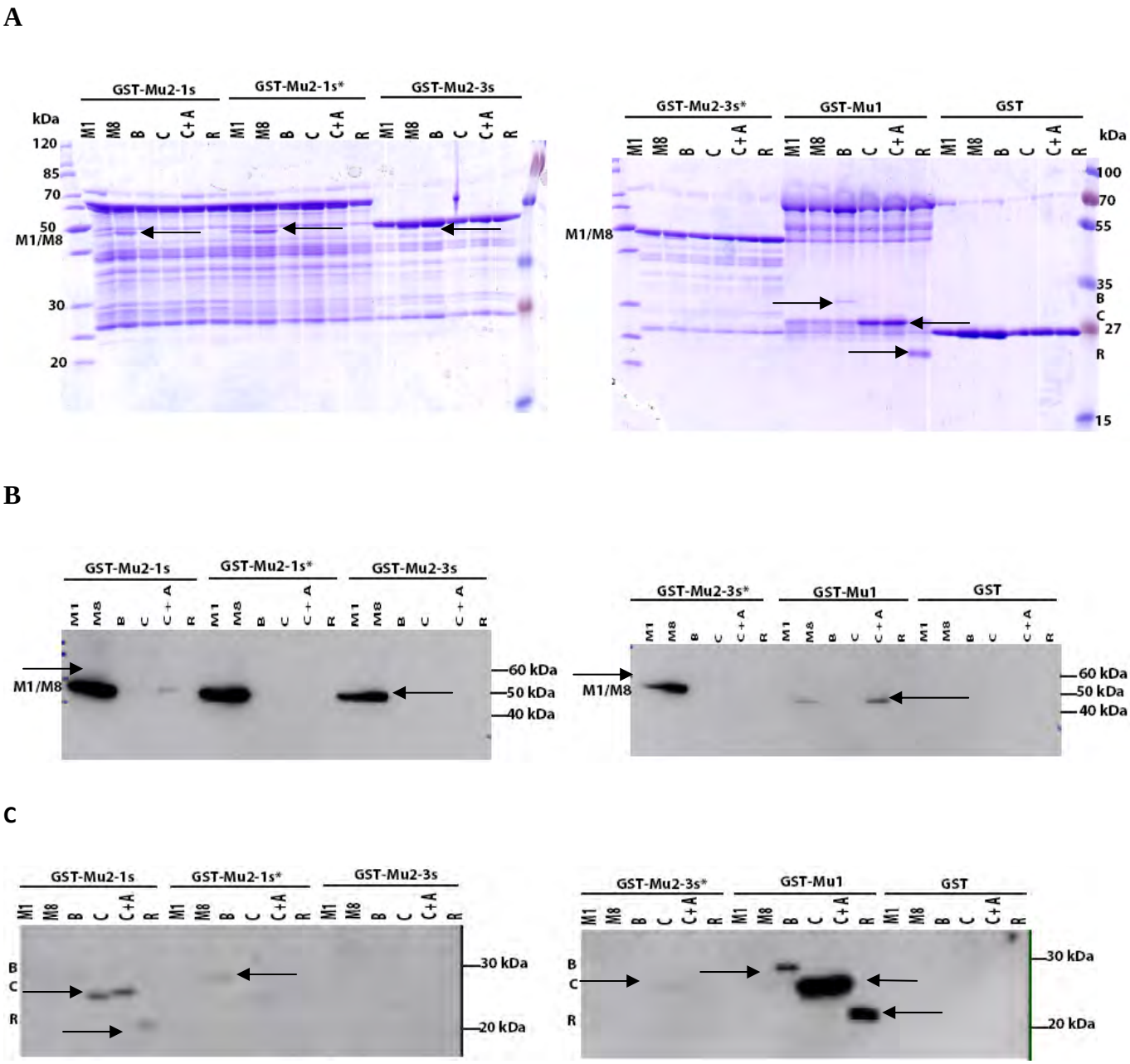


Fig. 2. GST-Mu2 interactions with the other active zone proteins. **(A)** 12.5% SDS-PAGE stained with Coomassie blue. Immobilized GST constructs: GST-Mu2-1s, GST-Mu2-1s_S385E (*), GST-Mu2-3s, GST-Mu2-3s_S385E(*), GST-Mu1 and GST, were incubated with mobile partners: Mu1pETcHis_K32E (M1), Mu1pETcHis_K32E,T117E (M8), HisBassoonH12-7 (B), HisCast3c (C), Thio-Acz7-3 (A) and HisRim5-8 (R), at room temperature and then protein-protein interactions were analyzed on SDS-PAGE. **(B)** Anti-cHis Immunodetection of GST-Mu2 interactions with other regulatory proteins of the active zone. GST-Mu1 and GST are positive and negative controls, respectively. **(C)** Anti-RGS Immunodetection of GST-Mu2 interactions with other active zone proteins (BassoonH12-7, Cast3c, Aczonin7-3 and Rim5/8). GST-Mu1 and GST are positive and negative controls, respectively. Arrows point to the proteins of interest and are aligned horizontally with the initial for the corresponding protein, for instance in Fig. 2A, the right pointing arrow in the bottom half of the gel indicates the position of Rim5-8, hereby marked by initial R. Asterisks (*) denote mutant variants.

Mu1 and other regulatory proteins of the active zone

Although Munc13-1-NT is known to interact with other active zone proteins, such as Aczonin, Bassoon, Cast, and Rim, the sequence of events associated with these interactions is yet to be determined (1-3, 6,7,10,12,14). I individually incubated His-BsnH12-7 (a.a. 3601-3820), Cast3c-5 (a.a. 415-619) and Rim 5-8 (a.a. 56-228), with immobilized GST-Mu1 (Munc13-1-NT, a.a. 2-320). A weak 30 kDa band corresponding to His-BsnH12-7, as well as stronger bands of 22 kDa (Rim 5-8) and 25 kDa (Cast3c), were detected (Fig. 2A,C). Interestingly, the 25kDa band attributed to Cast3c is of 1:1 stoichiometry with the GST-Mu1.

Reversing binding partners (Mu2 binds to immobilized Mu1)

As shown above, immobilized Mu2 variants (Munc13-1-NT, a.a. 363-651 and 363-507) were shown to interact with the dimerization mutant mobile partners Mu1pETcHis (K32E; K32E,T117E) (Munc13-1-NT, a.a. 1-320). In a complementary experiment, I reversed the immobilized and mobile partners, to confirm the specificity of the observed Mu2-Mu1 interactions. Indeed, immobilized GST-Mu1 constructs interacted with His-Mu2, as would be expected. A double band of 40 kDa, corresponding to His-Mu2-1s was detected on a 12.5% SDS-PAGE gel. Both the wild type GST-Mu1 and the dimerization mutant GST-Mu1 (T117E) precipitated the wild type His-Mu2 and phosphomimetic His-Mu2-1s (S385E) constructs (Fig. 3B). I concluded that the Mu1 part of the GST-fusion protein interacted with His-Mu2, because GST alone did not bind the mobile partner (Fig. 3B).

A

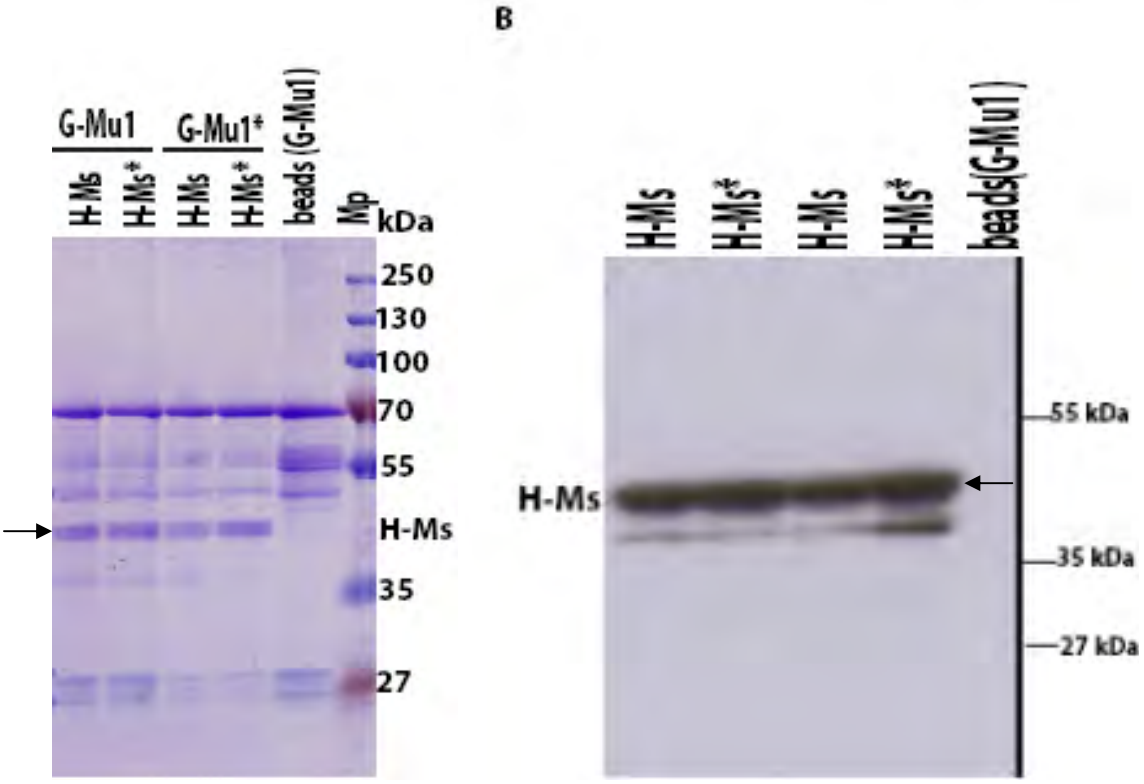


Fig. 3. (A) 12.5% SDS-PAGE of GST-Mu1, GST-Mu1_T117E(*), incubated with mobile partners: HisMu2-1s (H-Ms),His-Mu2-1s_S385E (H-Ms*) and stained with Coomassie blue. GST-Mu1 only is the negative control. Precipitation of His-Mu2-1s and His-Mu2-1s_S385E (H-Ms*) with GST-Mu1 and GST-Mu1_T117E(*), would result in a 40kDa band. **(B)** Imunodetection of GST-Mu1(*)-HisMu2-1s(*) interactions with anti-RGS-His antibody. Arrows point to the position of wildtype and mutant Mu2-1s marked as H-Ms, directly horizontal from the opposite side. Asterisks (*) denote mutant variants.

Phosphomimetic mutations T117E and S385E have no effect on Mu2-Mu1interactions

Phosphorylation of proteins at specific domains modifies protein-protein interactions and is a key regulatory feature in signal transduction (1). I introduced phosphomimetic mutations T117E and S385E to Mu1 and (Mu2-1s, Mu2-3s), respectively, in order to clarify the role of this phosphorylation site on Mu2-Mu1 interactions. Phosphomimetic mutations are expected to maintain constitutive phosphorylation at those sites. Both wild type (GST-Mu1) and phosphomimetic (GST-Mu1_T117E) constructs equally precipitated the two mobile His-Mu2 variants (Fig. 3B). Similarly, mobile Mu1pETcHis_K32E (M1) and Mu1pETcHis_K32E T117E (M8), were precipitated by the wild type and phosphomimetic (S385E) GST-Mu2 variants (a.a. 363-651 and a.a. 363-507) (Fig. 3 A, C).

Calmodulin abolishes interactions between GST-Mu1 and His-Mu2-1

A calmodulin binding site (CaM) is one of the features of Mu2 (Munc13-1-NT, a.a. 363-651) (Fig. 1A). I investigated the effect of pre-incubating His-Mu2-1s with 1 mM EGTA, 1 mM calcium and 2 µM calmodulin, on its intramolecular interaction with GST-Mu1 (a.a. 2-320). The characteristic 40kDa band attributed to His-Mu2-1s was abolished in the presence of calmodulin (Fig. 4A, B).

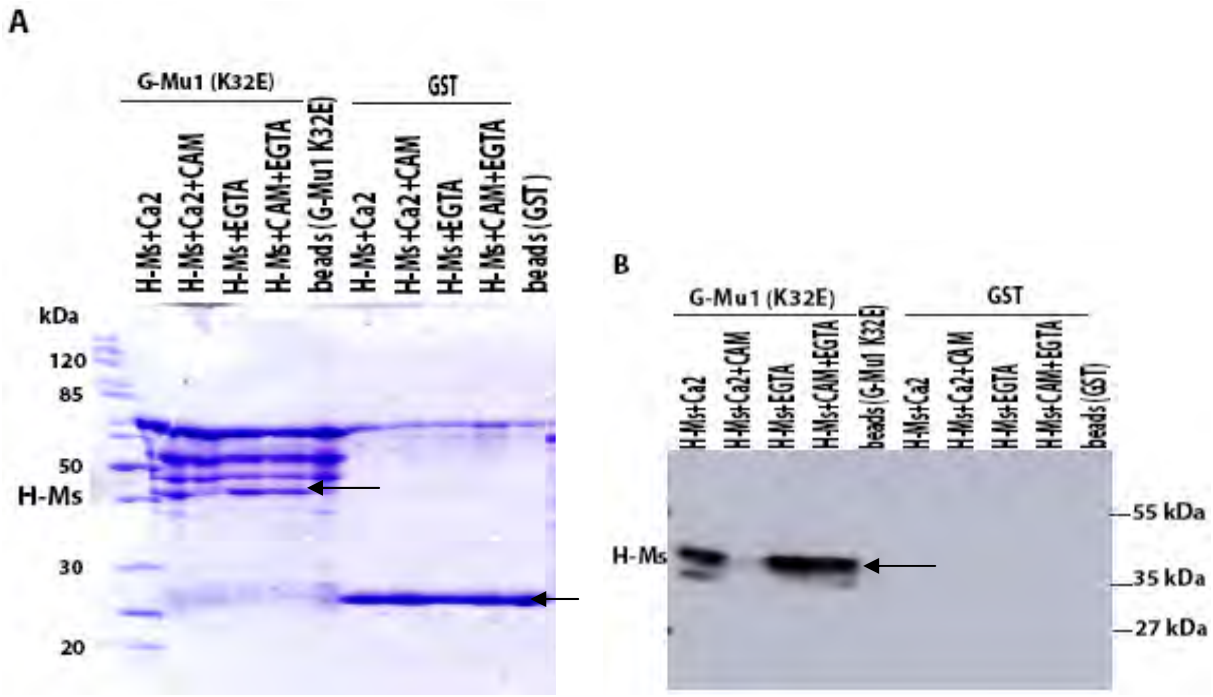


Fig. 4. The calmodulin effect on Mu1-Mu2 interactions. **(A)** Coomassie stain of GST-Mu1(K32E), GST and GST-Mu2-1s incubated with mobile partners: His-Mu2-1s (H-Ms), and Mu1pETcHis_K32E (M1), in the presence of 1 mM Ca2+ alone; 1 mM Ca2+ and 2 µM CaM; 1mM EGTA; and 1 mM EGTA and 2 µM CaM. **(B)** Anti-RGS Immunodetection of the above mentioned interactions. GST and GST-Mu2-1s were used as negative controls. Arrows indicate the positions of Mu2-1s (H-Ms) (top left oriented arrow, Fig. 4A and left pointing arrow Fig. 4B) and GST (bottom left oriented arrow, Fig. 4A).

DISCUSSION

Mu1-Mu2 Interactions

In this study I demonstrate an intramolecular interaction of the N-terminal region of Munc13-1 (designated, Munc13-1-NT, a.a. 1-320) with a downstream sequence mapped to a.a. 363-507, using pulldown assays and immunoblotting. Immobilized GST-Mu2-1s (a.a. 363-651) and it's shorter variant GST-Mu2-3s (a.a. 363-507) were both shown to interact with mobile Mu1pETcHis (a.a. 1-320). As such, the binding site of Mu1 was delineated to a.a. 363-507. Similar results were obtained when binding partners were reversed and immobilized Mu1 was incubated with mobile Mu2 variants. This finding suggests that Mu2 may fold back to bind Mu1.

Role of phosphomimetic mutations on Mu1-Mu2 Interactions

I also present evidence that introduction of phosphomimetic mutations T117E and S385E to Mu1 and Mu2 variants, respectively, has no effect on the observed intramolecular interaction of Munc13-1-NT. His-Mu1 was precipitated by either immobilized wild type or phosphomimetic GST-Mu2 variants (a.a. 363-507 and 363-651). Likewise, His-Mu2 variants were precipitated by both GST-Mu1 and GST-Mu1 (T117E). This suggests that the observed intramolecular interaction of Munc13-1-NT is not affected by introduction of phosphomimetic mutations (T117E or S385E) to Mu1 and Mu2, respectively. Other putative phosphorylation sites or post translational modifications (such as acetylation, methylation, or proteolytic cleavage) may be involved in modulating protein-protein interactions at Munc13-1-NT.

Current working model

Previous studies have shown Munc13-1-NT to be a center piece in a complex protein machinery that includes other active zone enriched proteins: Aczonin, Bassoon, Cast, and Rim (1-3,6,7,10,13,15). I confirmed a weak interaction between Mu1 and His-BsnH12-7, as well as stronger interactions with Rim 5-8 and Cast3c. These results indicate that Mu1 directly interacts with BsnH12-7, Cast3c and Rim 5-8 and support the current model (Kilimann M.W. and Limbach C, unpublished) in which Mu1 has binding domains for these proteins (Fig. 5).

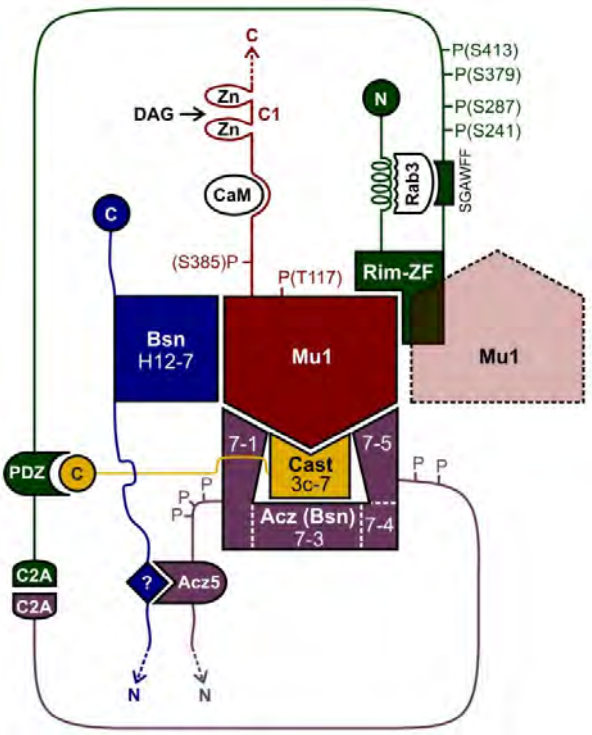


Fig. 5. Current working model: An illustration of the complex protein machinery involved in regulation of neurotransmitter exocytosis. Mu1(maroon) homodimerizes with another Mu1 subunit (grey), and also has binding domains for BsnH12-7(blue), Rim (green), Cast3c-7 (yellow), as well as Aczonin (purple). Cast3c-7 is shown to indirectly facilitate Acz7-3 interaction with Mu1. Aczonin extends to include a C2A domain, that may interact with a similar domain on Rim. Rim is shown to have a Rab3 binding site, putative phosphorylation sites: P(S413, S379, S287 and S241), as well as PDZ and C2A domains. Mu2 extends from Mu1 and appears as a 'maroon string' encompassing S385, CaM and C1 domains, as well zinc fingers (Zn). The N-terminal domain of Munc13-1 referred to in the thesis stretches from a.a. 1-651 to include Mu1 and Mu2 (maroon).

Role of Calmodulin on Mu1-Mu2 Interactions

Mu2 (Munc13-1-NT, a.a. 363-651) has a calmodulin binding site which is of particular interest because calmodulin has been suggested to mediate synaptic plasticity (20). As such, I was interested in determining the effect of calmodulin (CaM) on intra and inter-molecular interactions of Munc13-1-NT. In this study, the observed intramolecular interactions between Munc13-1-NT, a.a. 2-230 (Mu1) and Mu2 (a.a. 363-651), was abolished by pre-incubating His-Mu2-1s with 1 mM calcium and 2 µM CaM. The effect of calmodulin is especially interesting because neurotransmitter exocytosis is calcium dependent. Perhaps the intramolecular interaction between Mu1 and Mu2 is a critical step in serial events leading to neurotransmitter release. Calmodulin may be involved in regulating maturation of synaptic vesicles into a fusion-competent, primed state, prior to fusion with the pre-synaptic plasma

membrane. The calmodulin binding site was previously disrupted by introduction of a W460R missense mutation (2). I propose that the observed calmodulin effect should be relieved by introducing a similar mutation to Mu2-1s.

Taken together, my data show an intramolecular interaction of Munc13-1-NT whereby Mu2 may fold back to modulate interaction between two distant domains. Although the observed intramolecular interaction was not affected by introduction of phosphomimetic mutations (T117E and S385E) to Mu1 and Mu2, respectively, it was abolished by calmodulin. Future experiments should pursue the role of post-translational modifications on modulation of protein-protein interactions at the neurotransmitter release site. It would also be worthwhile to perform competition assays between Munc13 and the other active zone proteins. Moreover, site directed mutagenesis to disrupt interactions between Munc13 and the above mentioned interacting partners, should provide insight on the role of various domains which constitute the complex protein machinery, implicated in the modulation of neurotransmitter exocytosis.

MATERIALS AND METHODS

Strains and plasmids

Recombinant His tagged constructs

His-tagged constructs (Tables 6 and 7) were cloned and maintained in *E. coli* strain M15.pREP4, after which expression was done in SG13009.pREP4 (Qiagen). These strains lack a chromosomal copy of the *lacIq* mutation which is required for stable propagation of expression constructs encoding ”toxic” or hydrophobic proteins. High level *lac* repression is provided in trans by plasmid pREP4, which is maintained with selection on kanamycin. pREp4 is a pACYC derived plasmid that contains the p15 A replicon, confers kanamycin resistance and constitutively expresses the Lac repressor protein encoded by the *lacI* gene. Characteristics of the *E.coli* strains and expression plasmids are listed in Tables 1 and 2, respectively. His-tagged constructs were blunt-cloned at a SmaI site on pQE-32 (Qiagen). The plasmid includes several features: an optimized promoter-operator element consisting of phage *t5* promoter and two *lac* operator sequences, a synthetic ribosomal binding site, 6xHis-tag coding sequence, a multiple cloning site and translational stop codons in all reading frames, two strong transcription terminators, *β-lactamase* gene for ampicillin resistance, as well as ColE1 origin of replication . Protein expression under the control of the *t5* promoter is induced using isopropyl β-D-thiogalactoside (IPTG).

Table 1. Bacterial strains

E.coli strain	genotype or relevant characteristics	source/reference
DH5α	F$^-$, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rK$^-$, mK$^+$), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1	AmershamPharmacia
BL21	F$^-$,*ompT, hsdSB* (rB–, mB–), *gal*	AmershamPharmacia
M15.pREP4	F, *nal^s,str^s,tif^s,thi$^-$, lac, ara, gal, mtl$^-$, recA, uvr, lon*	Qiagen
SG13009.pREP4	F$^-$, *lac$^-$, ara , gal, mtl$^-$, recA, uvr*	Bio-Rad Inc.

F$^-$ indicates that the strain does not carry the F plasmid. *ompT* refers to a mutation in outer membrane protein protease VII that reduces proteolysis of expressed proteins. *recA* is a mutation in a gene responsible for general recombination of DNA, particularly desirable when cloning genes with direct repeats.

Table 2. Expression plasmids

plasmid	genotype or relevant characteristics	source/reference
pQE32	phage *t5* promoter, RBSII, 6xHis-tag sequence, *β-lactamase* gene, MCS	Qiagen
pREp4	internal *lacIq,* Kan^r, p15 A replicon	Qiagen
pGEX4T2	*tac* promoter, internal *lacIq*, factor Xa protease recognition site, MCS	AmershamPharmacia

RBSII refers to the synthetic ribosomal binding site for high translation rates. MCS is the multiple cloning site with translational stop codons in all reading frames for convenient preparation of expression constructs.

Glutathione-S transferase fusion constructs

GST-fusion constructs (Tables 6 and 7) were cloned and maintained in DH5α , then expressed in BL21 RIL, a codon plus strain (Table 1). GST-fusion constructs were blunt-cloned at a SmaI site on pGEX4T2 (AmershamPharmacia). The plasmid consists of the following features: a *tac* promoter for inducible high level expression, an internal *lacIq* for use in any *E. coli* host, and a PreScission thrombin or factor Xa protease recognition site for the desired

protein (Table 2). Protein expression is under the control of the *tac* promoter, which is induced using IPTG.

Medium

LB medium was prepared by dissolving 10 g Bacto Tryptone, 5 g yeast extract and 10 g NaCl in 800 ml H_2O. The pH was adjusted to 7.5 with NaOH and the volume adjusted to 1L with dH_2O. The medium was sterilized by autoclaving before use. Cultures were grown in LB supplemented with 50 µg/ml ampicillin .

DNA amplification

DNA was amplified using primers shown in Table 3 and reaction conditions in Table 4. Drop down polymerase chain reaction (PCR) with annealing at 68 ºC, 66 ºC, 64 ºC (5 cycles) and 62 ºC, 60 ºC, 58 ºC (10 cycles) was used. Initial denaturation: 98 ºC, 30s, 1 cycle; denaturation: 98 ºC, 15s; annealing: 68 ºC, 10s; extension: 72 ºC, 17s; final extension: 72 ºC, 7 min, hold at 4ºC. DNA was amplified from Mu1+2-1:pet, M4 and Mu1+2-1:petΔSmaI templates using Phusion Hot Start (a high fidelity DNA polymerase, from Finnzymes). Phusion buffer C was used in the PCR reaction (Tables 3 and 4). Blunt end cloning at a SmaI site was done in pQE-32 (Qiagen) and pGEX-4T2 (AmershamPharmacia) for His and GST-tagged constructs, respectively.

Table 3. Primers

Primer name	DNA Sequence
Mup1f	5’ TCTCTGCTGTGCGTGGGAGTC 3’
Mup2fB Munc13 aa295 Mus Forw	5’ AGGGACCGCGATTCTTATCA 3’
Mup2fsht Tm64 Mu13 aa364 Mus r	5’ CTGGCCAGCTACACCCAGC 3’
revMu1+2-1	5’ GAAGATCTCAGGCTTGTTGCG 3’
rev3Hpa1	5’ agttaacCTTCCGTTTCCTGATGTCT GG 3’
rev5Hpa1	5’ agttaacGA AACTCTCCTCAGACTTGGG 3’
rev3B Mu13 aa501 Tm62 Mus rev	5’ CTTCCGTTTCCTGAT GTCTGG 3’
T117E Mus mut	5’ CTGTGGGACCAAGGACCCGAGTTCCATCGCA TTCTCCTG 3’
S385E Mus mut	5’ CCAAAGAGTTCAAGCGGATCGAGTTCCCAAC GGCTGCGC 3’
08_ES_Munc-W460R	5’ CAGGGCCAAAGCCAACCGTTTGCGAGCCTTC AACA 3’

Primer names and their corresponding DNA sequences are shown. Mup1f, Mup2fB and Mup2fsht are forward primers, revMu1+2-1, rev3HpaI, rev5HpaI and rev3B are reverse primers, whereas T117E, S385E and W460R are site directed mutagenesis primers.

Table 4. PCR reaction mix

Ingredient	Source	Stock	Volume
deionized water	N/A	N/A	35 µl
Phusion buffer C	Finnzymes	10x	5 µl
dNTP mix	Qiagen	2.5 mM	3 µl
Forward, Reverse primers	Invitrogen	20 µM	2.5 µl
DNA template	N/A	0.3215 µg/ µl	1 µl
Phusion DNA polymerse	Finnzymes	5 U/µl	1 µl

PCR ingredients and stock solutions were added in the same order as above.

Site directed mutagenesis

Phosphomimetic mutations (T117E, S385E) and a W460R mutation at a calmodulin binding site (CaM) were introduced by the Quick Change Multi Site-Directed Mutagenesis Kit, (Stratagene) using primers shown in Table 3. Samples were sequenced at Uppsala genomic center.

Purification of amplified PCR products

PCR reaction components were removed from the amplified PCR products prior to use in cloning. A PCR purification kit (QIAquick, Qiagen) was used. Five volumes of buffer PB were applied to a single volume of PCR sample, mixed and placed in a spin column with a 2 ml collection tube. The column was spun at 10,000g for 30-60 seconds, after which flow-through was discarded and column was retained in the collection tube. Column was washed with 0.75 ml buffer PE and previous step (centrifugation and discarding flow-through) was repeated. Column was spun for an extra 60 seconds, before transfer to a new 1.5 ml tube and eluting DNA with 50 µl of water.

Blunt Cloning

His and GST-tagged constructs were blunt cloned in pQE-32 (Qiagen) and pGEX-4T2 (AmershamPharmacia) plasmid vectors digested with SmaI at the multiple cloning site

(MCS), respectively. In the reaction, 1:5 of plasmid vector (25 ng) : insert (target DNA) (100 ng) was used (Table 5).

Table 5. Ligation reaction mix

Ingredient	Source	construct	negative control
Y-tango buffer (10x)	Fermentas	1 µl	1 µl
ATP (10 mM)	Fermentas	0.5 µl	0.5 µl
dNTP mix (2mM)	Fermentas	0.5 µl	0.5 µl
Plasmid vector* (50ng/µl)	Qiagen/Invitrogen	0.5 µl	0.5 µl
SmaI (10U/ µl)	Fermentas	0.1 µl	0.1 µl
T4-DNA ligase (5U/ µl)	Fermentas	0.25µl	0.25 µl
Insert (target DNA) (20 ng/µl)	N/A	5 µl	_
Deionized water	N/A	2.15 µl	7.15 µl

*plasmid vectors used were pQE-32 (his- tagged constructs, Qiagen) and pGEX-4T2 (GST- tagged constructs, Invitrogen). Ligation reaction was performed at room temperature for 1 hour.

Transformation of recombinant constructs in E. coli competent cells by the calcium chloride method

At 4 ºC, 100 µl *E. coli* competent cells were transferred into a chilled tube to which 2.5 µl of the ligation reaction mix (Table 5) was added, thoroughly mixed by gentle swirling and incubated on ice for 30 minutes. The mix was then heat shocked at 42 ºC (water bath) for 90 seconds in a circulating water bath**,** after which it was chilled on ice for 2 minutes. Transformed cells were then supplemented with 0.9 ml LB, 5% glucose in growth tube with loosened cap, and incubated at 37 ºC for 1 hour with shaking. Plating of the cells involved spreading 200 µl of the transformed cells on LB plates supplemented with 50 µg/ml ampicillin. Plates were incubated at 37 ºC for 16 hours.

Screening

Preliminary screening of recombinant constructs (Tables 6 and 7) was by colony PCR. Forward primers of corresponding plasmid vectors were used (pGEXp5' and pQE5III) in combination with reverse primers of individual sequence regions (revMu1+2-1, rev3B,

rev3HpaI) (Table 3). Constructs of interest were subsequently verified by small scale test expression and sequencing at the Uppsala Genomic Center. In the small scale expression, 4 ml cultures were grown overnight in LB supplemented with 50 µg/ml ampicillin at 37 ºC until OD_{600} = 0.6, before induction with IPTG for 3 hours at 37ºC. Protein purification was done as in the large scale expression (see procedure below), except 0.1 volume of reagents was used. Samples were also eluted directly from beads by adding 20 µl of 2x loading dye (see gel electrophoresis). The same forward primers and corresponding reverse primers (mentioned above) were used in the sequencing reactions (Table 3.)

Table 6. Munc13 cDNA expression constructs made in the course of this study.

	Name	Template	F:primer	R:primer	Length (kb)	Length (aa)	Predicted size (kDa)
1	His-Mu2-1s	Mu1+2-1:pet	Mup2fsh	revMu1+2-1	0.864	289aa, 363DLA…651EIF	35.1kDa
2	His-Mu2-3s	Mu1+2-1:pet	Mup2fsh	rev3HpaI	0.432	145aa, 363DLA…507KRK	18.9kDa
3	His-Mu2-3s (S385E)	M4	Mup2fsh	rev3HpaI	0.432	145aa, 363DLA…507KRK	18.9kDa
4	GST-Mu2-1s	Mu1+2-1:pet	Mup2fsh	revMu1+2-1	0.864	289aa, 363DLA….651EIF	58.1kDa
5	GST-Mu2-3s	Mu1+2-1:pet	Mup2fsh	rev3HpaI	0.432	145aa, 363DLA…507KRK	41.9kDa
6	GST-Mu2-3s (S385E)	M4	Mup2fsh	rev3HpaI	0.432	145aa, 363DLA…507KRK	41.9kDa
7	His-Mu2-1s (S385E)	Mu1+2-1:pet	Mup2fsh	revMu1+2-1	0.864	289aa, 363DLA....651EIF	35.1kDa
8	GST-Mu2-1s (S385E)	Mu1+2-1:pet	Mup2fsh	revMu1+2-1	0.864	289aa, 363DLA...651EIF	58.1kDa

His and GST-tagged constructs were numbered (1-6, 14,17) and (7-13,15-16 and 18), respectively. The templates used were: Mu1+2-1:pet, M4, and Mu1+2-1:pe ΔSmaI, which are based on cDNA from mouse. Brackets below construct names (column 2), denote mutations of interest, for instance (S385E), refers to an amino acid substitution of a serine 385 for a glutamate. Columns 4 and 5 show the forward and reverse primers used respectively, whereas the amino acid length of the constructs is indicated in column 7. Letters in column 7 refer to amino acids, for instance row 3, column 7 (**363DLA…651EIF),** implies that the starting amino acid of this construct is D (aspartic acid) and the final amino acid is F (phenylalanine).

Table 7. Other active zone protein expression constructs, previously made in the Kilimann laboratory

	Name	Template or accesion number	F:primer	R:primer	Length (kb)	Length (aa)	Predicted size (kDa)
1	Mu1pETcHis (K32E)	NM_021468			.954	319aa, 2SLL…320QDE	39.4kDa
2	Mu1pETcHis (K32E, 117E)	NM_021468			.954	319aa, 2SLL…320QDE	39.4kDa
3	GST-Mu1	NM_021468			.954	319aa, 2SLL…320QDE	62.4kDa
4	GST-Mu1(T117E)	NM_021468			.954	319aa, 2SLL…320QDE	62.4kDa
5	His-BsnH12-7	NM_019146			0.660	220aa, 3601SDR….3820SQP	27kDa
6	His-Cast3c-7	AAR_14795			0.216	72aa, 506LNK…577DSS	11.4kDa
7	His-Cast3c-5	AAR14795			0.336	113aa, 465SDC…577DSS	16.2kDa
8	His-CAM	RT – cDNA, Siv L11-7-7-16.1 brain wt	Calmodulin mus forw nt1	Calmodulin mus rev end	0.450		20kDa
9	Thio-Acz7-3	NM_011995				59aa, 3657RAK…3715ERE	21.1kDa
10	GST-BsnH12-7	NM_019146			0.660	220aa, 3601SDR…3820SQP	50kDa
11	GST-Rim5/8	AJ_310531				169aa, 56CVV…228EVP	45.3kDa
12	GST-Acz7-6	NM_011995				192aa, 3593SRA…3784PYT	47.9kDa
13	GST-Acz7-3	NM_011995				59aa, 3657RAK…3715ERE	33.1kDa

His and GST-tagged constructs were numbered (1-8 and 10) and (10-14), respectively. The templates used were based on cDNA from mouse.

Gel electrophoresis

Separating sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Table 8) were made at a concentration of 12.5% in 0.25 M Tris-HCl pH 8.8, whereas the stacking gels (Table 9) were at a concentration of 4.5% in 0.125 M Tris-HCl pH 6.8. SDS-PAGE (2x) loading buffer dye was added to protein samples prior to electrophoresis on SDS-PAGE gels. The loading buffer dye contained 25 % SDS, 62.5 mM Tris-HCl (pH 6.8), 25% gylcerol, and 0.01% bromophenol blue. Gels were cast using biorad gel apparatus and run with 1x SDS-PAGE running gel buffer **(**0.25 M Tris, 1.92 M glycine 1.0 % SDS pH

8.3), at 100 V for 150 minutes. Staining of gels was in 0.1 Coomassie Blue R-250 staining solution (40 % methanol, 10 % acetic acid, 0.1 % Coomassie Blue R-250 and 50 % water), for 45 minutes at room temperature, with shaking. Gels were finally destained in Coomassie Blue R-250 destaining solution (40 % methanol, 10 % acetic acid, and 50 % water), under similar conditions as in previous step prior to scanning.

Table 8. Separating SDS-PAGE gels

Ingredient	Volume (30 ml)	Volume (10ml)
40 % acrylamide stock*	9.4 ml	3.1 ml
water (distilled)	12.72 ml	4.24 ml
1 M Tris-HCl pH 8.8	7.5 ml	2.5 ml
10 % SDS	0.3 ml	0.1 ml
10 % APS	50 µl	50 µl
TEMED (added last)	10 ul	10 ul

* = 19:1 - 38:1 w:w ratio of acrylamide to N,N'-methylene bis-acrylamide . Separating SDS-PAGE gels are usually kept overnight at 4 ºC, before use.

Table 9. Stacking SDS-PAGE gels

Ingredient	Volume (15 ml)	Volume (10 ml)
40 % acrylamide stock*	1.7 ml	1.1 ml
water	11.4 ml	7.5 ml
1 M Tris-HCl pH 6.8	1.9 ml	1.25 ml
10% SDS	0.15 ml	0.1 ml
10% APS	50 µl	50 µl
TEMED (stir quickly)	10 µl	10 µl

* = 19:1 - 38:1 w:w ratio of acrylamide to N,N'-methylene bis-acrylamide .

High level expression of recombinant constructs

Bacterial cultures were inoculated with 1:50 overnight seed cultures, grown in LB supplemented with ampicillin, at 37ºC until OD_{600} = 0.6 and induced with 1 mM IPTG for 3 hours, at 37ºC. Cultures were harvested by centrifugation at 33,000g for 20 minutes, the supernatant was decanted and pellets immediately frozen at -70 ºC.

Purification of GST- tagged proteins at 4º C

Bacterial pellets were thawed on wet ice for 15-20 minutes, resuspended in Tris buffer (20 mM TrisHCl, 150 mM NaCl, 1%Triton X-100, pH 7.5) and incubated on wet ice for 30 minutes, with shaking. Cells were then sonicated thrice on wet ice (settings 5-5), at 10 sec intervals. Centrifugation was done at 9000g for 15 minutes at 4 ºC. GSH-agarose beads (Amersham Pharmacia) (50% slurry), equilibrated in PBS-D buffer (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4*2H_2O$, 1.5 mM NaH_2PO_4, pH 7.5) for 4 x 10 minutes at room temperature were added to the supernatant, which was then incubated at 4 ºC for 45 minutes with head over rotation. Samples were centrifuged at 9000g for 15 seconds and the supernatant aspirated by a vacuum pump. Beads were handled carefully to avoid drying, washed thrice with Tris buffer and adjusted to 50% slurry, in the same buffer. The 50% slurry was blocked overnight in 5% BSA-Tris buffer at 4 ºC, before use in a GST pull-down assay.

Native purification of His- tagged proteins at 4ºC

Bacterial pellets were thawed on wet ice for 15-20 minutes, after which they were resuspended in lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, 1% Triton X-100, pH 8.0) supplemented with aprotinin (2 µg/µl), pepstatin A (1 µg/µl), leupeptin (2 µg/µl) and incubated on wet ice for 30 minutes, with shaking. Cells were then sonicated thrice on wet ice (settings 5-5), at 10 second intervals. Centrifugation was done at 9,000g for 15 minutes. Ni-NTA-agarose beads (50% slurry), equilibrated in lysis buffer (4 x 10 minutes, at room temperature) were added to the supernatant, which was incubated at 4 ºC, for 45 minutes with head over rotation. Samples were centrifuged at 9000g for 15 seconds and the supernatant aspirated by vacuum pump. The beads were washed thrice with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole, 1% Triton X-100, pH 8.0), proteins eluted twice by applying one volume of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, 1% Triton X-100, pH 8.0) and incubating twice at 4 ºC for 20 minutes. The resulting supernatant was analyzed on 10-15% SDS-PAGE, depending on the predicted size of protein, and stored at 4 ºC, before use in a GST pull-down assay.

Denaturing purification of His- tagged proteins at room temperature

Some His-tagged proteins (His-Mu2-1s, His-Mu2-3s) which were sequestered in inclusion bodies were purified under denaturing conditions. Bacterial pellets were thawed on wet ice for 10 minutes, resuspended in buffer B (100 mM NaH_2PO_4, 10 mM TrisHCl, 8 M urea, pH 8.0)

and incubated at room temperature for 45 minutes, with shaking. Cells were then sonicated thrice on wet ice (settings 5-5), at 10 second intervals. Centrifugation was done at 9000g for 20 minutes. Ni-NTA-agarose beads (50% slurry), equilibrated in buffer B (4 x 10 minutes, at room temperature), were added to the supernatant, which was incubated at room temperature, for 45 minutes with head over rotation. Samples were centrifuged at 9000g for 15 seconds and the supernatant aspirated by vacuum pump. Beads were washed thrice with buffer C (100 mM NaH_2PO_4, 10 mM TrisHCl, 8 M urea, pH 6.3), proteins eluted twice by applying one volume of buffer E (100 mM NaH_2PO_4, 10 mM TrisHCl, 8 M urea, pH 4.5) and incubating twice at room temperature for 30 minutes. The resulting supernatant was stored at room temperature.

Pull-down assays

Soluble proteins were mixed in pull-down (PD) buffer (20 mM Tris HCl, p.H 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with aprotinin (2 µg/µl), pepstatin A (1 µg/µl), leupeptin (2 µg/µl), 1 mM dithiothreitol (DTT) and 0.5% bovine serum albumin (BSA). Samples were kept on ice during pipetting. GST tagged proteins were added and properly mixed prior to incubation at room temperature for 2 hours. Samples were spun at 5200g for 30 seconds, and supernatant was aspirated. The beads were washed thrice with ice cold PD buffer without protease inhibitors. The wash step included incubation at room temperature for 90 seconds, with shaking. Finally, the sample was centrifuged and supernatant was carefully aspirated and 2x loading dye added to elute the beads. The amount of loading dye added depended on the number of desired aliquots. In this study I used seven aliqouts and therefore maintained a total volume of 28 µl at the elution step. Samples for SDS PAGE analysis were boiled for 3 minutes and stored at -20ºC.

Western blotting

Proteins were subjected to 12.5% SDS-PAGE and subsequently transferred to a 0.45µM nitrocellulose transfer membrane (Whatman), prior to probing with RGS-His (Qiagen), cHis (Invitrogen) and mouse HRP conjugated (Invitrogen) antibodies (Table 10). Transfer of proteins from SDS-PAGE to the nitrocellulose membrane was done by the semi-dry method. Nine Whatman papers proportional to the gel size were used to make a sandwich where three of each were wet with buffers C (25 mM Tris HCl pH 9.2, 40 mM aminohexoacid, and 20% methanol, v/v)), B (30 mM Tris HCl pH 10.4, 20% methanol, v/v) and A (300 mM Tris HCl pH 10.4, 30% methanol, v/v). The gel was immersed in buffer C and a nitrocellulose membrane in buffer B. The sandwich from bottom to top, included: three Whatman papers

wet with buffer C, the gel, followed by nitrocellulose membrane immersed in buffer B, three Whatman papers wet with buffer B and finally three Whatman papers wet with buffer A. Tweezers were carefully rolled over the sandwich to get rid of bubbles. Immunotransfer was done at 50 mA/gel for 1 hour. The nitrocellulose membrane was removed, stained with

Ponceau by shaking at room temperature for 3-5 minutes, labeled and scanned. After destaining in water by shaking at room temperature for 3-5 minutes with addition of 3-5 drops of 1M Tris-HCl, pH 7.5, the membrane was blocked overnight at 4ºC in blocking solution (10% powder milk, 0.1% Tween20 in TBS), to reduce unspecific background. TBS (137 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl and 40 μM phenol red, pH 7.4, (buffered in HCl).

Table 10. Antibodies used for Western blotting

Antibody	Vendor
Mouse anti-His C terminal antibody	Invitrogen
Mouse anti-RGS-His antibody	Qiagen
ECL Peroxidase labeled anti-mouse IgG antibody	Invitrogen

Mouse anti-His C terminal antibody was used to detect constructs with C-terminal His tag, whereas those with N-terminal His tag were detected by mouse anti-RGS-His antibody. ECL Peroxidase labeled anti-mouse IgG antibody was the secondary antibody.

Immunodectection using anti-RGSHis antibody

Blots were washed twice in TBS for 10 minutes at room temperature, with shaking. The anti-RGS antibody was used for immunodetection of proteins with an N-terminal His tag. It was diluted at 1:2000 v/v in 3% BSA-TBS and incubated for 1 hour at room temperature, with shaking. The wash step was repeated prior to addition of a secondary antibody (anti-mouse HRP conjugated, 1:10000), which was diluted in 10% milk, 0.1% Tween 20-TBS, for 45 minutes at room temperature. The nitrocellulose membrane was finally washed four times in 0.1% Tween 20-TBS for 10 minutes at room temperature. Signals were detected by enhanced chemiluminescence with the ECL plus detection kit (Amersham). The blot was developed on a high performance chemiluminescence film (Amersham Biosciences) and exposed at different intervals: (30 seconds-10 minutes).

Immunodectection using anti-cHis antibody

The membrane was washed twice in 0.05% Tween 20 in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4), for 5 minutes, with shaking. Anti-cHis antibody (Table 9) was used for immunodetection of proteins with C-terminal His tag. It was diluted at 1:5000 v/v in 5% powder milk, 0.1% Tween 20 in PBS and incubated for 1 hour at room temperature with shaking. The wash step was repeated prior to addition of a secondary antibody (anti-mouse HRP conjugated, 1:10000), which was diluted in 10% milk, 0.1% Tween 20 in TBS, for 45 minutes at room temperature. The wash step was repeated prior to signal detection. The film was exposed at intervals ranging from 1 to 60 minutes.

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