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Characterization of two distinct binding modes between syntaxin 4 and Munc18c

Veronica ARAN, Fiona M. BRANDIE, Alasdair R. BOYD, Theodoros KANTIDAKIS, Elizabeth J. RIDEOUT, Sharon M. KELLY, Gwyn W. GOULD and Nia J. BRYANT[1]

Henry Wellcome Laboratory of Cell Biology, Division of Molecular and Cellular Biology, Davidson Building, Faculty of Biomedical and Life Science, University of Glasgow, Glasgow G12 8QQ, U.K.

Interaction of SM (Sec1/Munc18) proteins with their cognate syntaxins represents an important regulatory mechanism of SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor)-mediated membrane fusion. Understanding the conserved mechanisms by which SM proteins function in this process has proved challenging, largely due to an apparent lack of conservation of binding mechanisms between different SM–syntaxin pairs. In the present study, we have identified a hitherto uncharacterized mode of binding between syntaxin 4 and Munc18c that is independent of the binding mode shown previously to utilize the N-terminal peptide of syntaxin 4. Our data demonstrate that syntaxin 4 and Munc18c interact via two distinct modes of binding, analogous to those employed by syntaxin 1a–Munc18a and syntaxin 16–Vps45p (vacuolar protein sorting 45). These data support the notion that all syntaxin/SM proteins bind using conserved mechanisms, and pave the way for the formulation of unifying hypotheses of SM protein function.

Key words: membrane fusion, Munc18c, Sec1/Munc18 protein (SM protein), soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor (SNARE), syntaxin 4, vacuolar protein sorting 45 (Vps45p).

INTRODUCTION

SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) proteins are central to the regulation of membrane trafficking in all eukaryotic systems, providing not only the energy to drive bilayer fusion, but also a degree of specificity to the process [1]. Although it has been established that formation of a SNARE complex, comprising three coiled-coil SNARE motifs contributed by the t-SNARE (target SNARE) (one from a syntaxin protein and two from light-chain SNAREs) on the target membrane and another from the v-SNAREs (vesicle SNAREs) on the donor vesicle, is sufficient to drive membrane fusion *in vitro*, it is clear that other factors are required for the regulation of this process in the cell. One family of proteins that are central to the regulation of SNARE complex formation are the SM (Sec1/Munc18) proteins, most of which bind to their cognate syntaxins with high affinity [2]. Understanding the mechanism(s) by which SM proteins function has proved problematic largely due to seemingly conflicting data concerning the way in which different SM family members interact with their cognate syntaxins [2,3].

Structural studies have revealed that the neuronal Sx1a (syntaxin 1a) adopts two distinct conformations [4,5]. In the closed conformation, the autonomously folded N-terminal regulatory Habc domain is folded back on to the juxtamembrane SNARE motif, rendering it unavailable for SNARE complex formation. In the open conformation, the Habc domain is moved away from the SNARE motif, leaving it free to participate in the core complex. Munc18a was originally identified as an Sx1a-binding protein whose binding to Sx1a precludes SNARE complex formation [6,7]. Consistent with this, crystallographic studies revealed that Munc18a is an arch-shaped molecule that cradles monomeric Sx1a in its closed conformation with contacts being made between the inner arch of the SM protein and almost the entire length of the syntaxin's cytosolic domain [5]. These data support a model in which SM proteins bind their cognate syntaxins in a closed conformation and regulate SNARE complex assembly, perhaps facilitating a switch of syntaxins from their closed to their open conformation [4].

In striking contrast with the interaction between Munc18a and Sx1a captured by the crystal structure [5], the extreme N-terminal region, preceding the Habc domain, of other syntaxins is both necessary and sufficient to capture their cognate SM proteins [8–12]. For example, the N-terminal 44 residues of Sed5p are sufficient to bind the SM protein Sly1p [8,13]. The crystal structure of this interaction reveals that the N-terminal peptide of the syntaxin inserts into a hydrophobic pocket on the outer face of the SM protein [8]. This 'pocket mode' of binding is consistent with the SM protein binding to either the closed or open conformations of the syntaxin.

The finding that different SM proteins bind their cognate syntaxins via strikingly different mechanisms has severely hampered formulation of a unifying hypothesis describing the mechanisms by which SM proteins regulate SNARE-mediated membrane fusion. We have recently demonstrated that the yeast SM protein Vps45p (vacuolar protein sorting 45) uses two distinct modes of binding to interact with its cognate SNARE proteins at different stages of the SNARE complex assembly/disassembly cycle [14,15]. Vps45p dissociates from its monomeric syntaxin, Tlg2p, before *trans*-SNARE complex formation and then reassociates following membrane fusion and the conversion of *trans*-SNARE complexes into *cis*-SNARE complexes [14,15]. We hypothesize that all syntaxin–SM pairs interact using both of these modes of binding at different stages of the SNARE assembly/disassembly cycle. In this model, the SM protein would prevent futile reformation of *cis*-SNARE complexes following the action of

Abbreviations used: GST, glutathione transferase; IPTG, isopropyl β-D-thiogalactoside; SM, Sec1/Munc18; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor; Sx1a, syntaxin 1a; Sx4, syntaxin 4; Vps45p, vacuolar protein sorting 45.

[1] To whom correspondence should be addressed (email n.bryant@bio.gla.ac.uk).

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the ATPase NSF (*N*-ethylmaleimide-sensitive fusion protein) and allow the SNARE proteins to recycle for further rounds of membrane fusion.

Consistent with the above, and in contrast with the long-held contention that Munc18a binds only to the closed conformation of Sx1a [16], it has recently been demonstrated that Munc18a also binds to the syntaxin's extreme N-terminus [17–19] and also to the assembled SNARE complex [20]. Importantly, deletion of the N-terminal 21 residues from a mutant version of Sx1a locked in the open conformation severely diminished binding of Munc18a, indicating that, like Vps45p, Munc18a uses distinct modes of binding to interact with its cognate SNARE proteins [17–19].

In the present study, we sought to characterize the binding between Munc18c and Sx4 (syntaxin 4), an interaction that mediates the insulin-stimulated delivery of GLUT4 (glucose transporter 4) vesicles to the plasma membrane of adipocytes and muscle cells (reviewed in [21]). Recent crystallographic studies have revealed that the binding of Munc18c to Sx4 is analogous to the interaction captured by the Sly1p/Sed5p crystal structure, with the N-terminus of Sx4 inserting into a hydrophobic pocket on the outer surface of the SM protein [11]. In the present study, we have used an *in vitro* binding approach to establish that Sx4 can interact with Munc18c, not only through this pocket mode of binding as described previously, but also through a distinct, previously uncharacterized, mode.

EXPERIMENTAL

Plasmids

N-terminally tagged Sx4 species

Plasmid pGEX5-Sx4 encoding the cytosolic domain of Sx4 (residues 1–273) tagged at the N-terminus with GST (glutathione transferase) was constructed using PCR to amplify the Sx4-coding sequence from a cDNA provided by Richard Scheller (Genentech, San Francisco, CA, U.S.A.). The product of this reaction was subcloned into pGEX-5X1. The open mutant (L165A, E166A), the NΔ mutant (residues 37–273) and the NΔ-open mutant (residues 37–273, L165A, E166A) were then generated from this plasmid by PCR and/or site-directed mutagenesis.

C-terminally tagged Sx4 species

To create a plasmid allowing expression of C-terminally tagged GST-fusion proteins, the coding sequence for GST was amplified from pGEX-5X1 with XhoI and PacI restriction sites at either end. This was then used to replace the S-tag of pETDuet-1 (Novagen). Sx4 species were amplified from the plasmids described above and inserted in-frame with the GST tag in pETDuet-1-GST. The L8K mutants were generated using site-directed mutagenesis.

Purification of GST-fusion proteins and Munc18c

GST-fusion proteins were expressed in Rosetta 2(DE3) *Escherichia coli* cells. Protein production was induced at 30 °C overnight by the addition of 0.5 mM IPTG (isopropyl β-D-thiogalactoside). *E. coli* were harvested by centrifugation at 4000 ***g*** for 20 min and lysed in GST purification buffer (100 mM Hepes, pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$ and 2 mM 2-mercaptoethanol) containing Complete™ protease inhibitors (Roche), by the addition of lysozyme and sonication. GST-tagged proteins were purified using glutathione–Sepharose (GE Healthcare). Unbound protein was removed by washing with PBS. Bound protein was eluted from the beads with 50 mM Tris/HCl (pH 8) and 15 mM glutathione. Munc18c was expressed as an N-terminal His_6-fusion protein from the vector pQE30 in M15 cells co-transformed with a vector encoding GroEL. Protein production was induced at 22 °C overnight by the addition of 0.2 mM IPTG. *E. coli* were harvested by centrifugation at 4000 ***g*** for 20 min and lysed in His_6-purification buffer [25 mM Hepes (pH 7.4), 200 mM KCl, 10 % (w/v) glycerol, 2 mM 2-mercaptoethanol and 15 mM imidazole] containing EDTA-free Complete™ protease inhibitors (Roche), by the addition of lysozyme and sonication. His_6-tagged protein was purified using Ni-NTA (Ni^{2+}-nitrilotriacetate) resin (Qiagen). Unbound protein was removed from the resin by washing with His_6-purification buffer. Bound protein was eluted from the resin with His_6-elution buffer [25 mM Hepes (pH 7.4), 200 mM KCl, 10 % (w/v) glycerol, 2 mM 2-mercaptoethenol and 400 mM imidazole]. Purified protein was dialysed against PBS and analysed by SDS/PAGE (12 % gels) and Coomassie Blue staining.

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GST pull-downs

A 5 μg sample of GST or GST-tagged protein was incubated with 10 μl of glutathione–Sepharose (50 % slurry) in a volume of 100 μl of binding buffer (20 mM Hepes, pH 7.4, 150 mM potassium acetate, 1 mM $MgCl_2$ and 0.05 % Tween 20) for 1 h in the cold room with end-over-end rotation. Unbound protein was removed by washing the beads three times with binding buffer, and beads were collected by centrifugation at 500 ***g*** for 2 min at 4 °C. Then, 15 μg of Munc18c was added in a total volume of 500 μl of binding buffer and was incubated overnight at 4 °C with continual mixing. Unbound protein was removed by washing three times with binding buffer containing 1 mg/ml fish-skin gelatin, followed by three washes with binding buffer containing 5 % (w/v) glycerol, then three washes with binding buffer alone. After the final wash, the beads were resuspended in 15 μl of SDS/PAGE sample buffer and heated to 95 °C for 5 min before analysis by SDS/PAGE (12 % gels) and Coomassie Blue staining. Binding was quantified by densitometric scanning of non-saturating exposed films using TotalLab imaging software (Phoretix).

Circular dichroism

CD measurements were obtained using a Jasco J-810 spectropolarimeter. For near-UV CD analysis, data were collected from 320 to 250 nm in a 0.5-cm-pathlength quartz cuvette using a protein concentration of 1 mg/ml, a scan rate of 10 nm/min, a bandwidth of 1 nm and a response time of 2 s. For far-UV CD analysis, data were collected from 260 to 180 nm in a 0.02-cm-pathlength quartz cuvette using a protein concentration of 0.3 mg/ml, a scan rate of 50 nm/min, a bandwidth of 1 nm and a response time of 0.5 s. Sx4 and Sx4-open were cleaved from the GST tag using thrombin before analysis.

Protease digestion experiments

Recombinant wild-type Sx4 or Sx4-open were incubated with chymotrypsin (1:100, w/w) in PBS and, at the time points indicated, samples were taken and immediately boiled in an equal volume of 2× SDS/PAGE sample buffer. Samples were then analysed by SDS/PAGE (12 % gels) and Coomassie Blue staining.

Surface plasmon resonance

Interactions between Munc18c and the GST-tagged Sx4 constructs were assessed at 25 °C using a Biacore 2000 instrument (Biacore AB). Syntaxin constructs were immobilized on to a CM5 sensorchip via anti-GST antibodies covalently coupled

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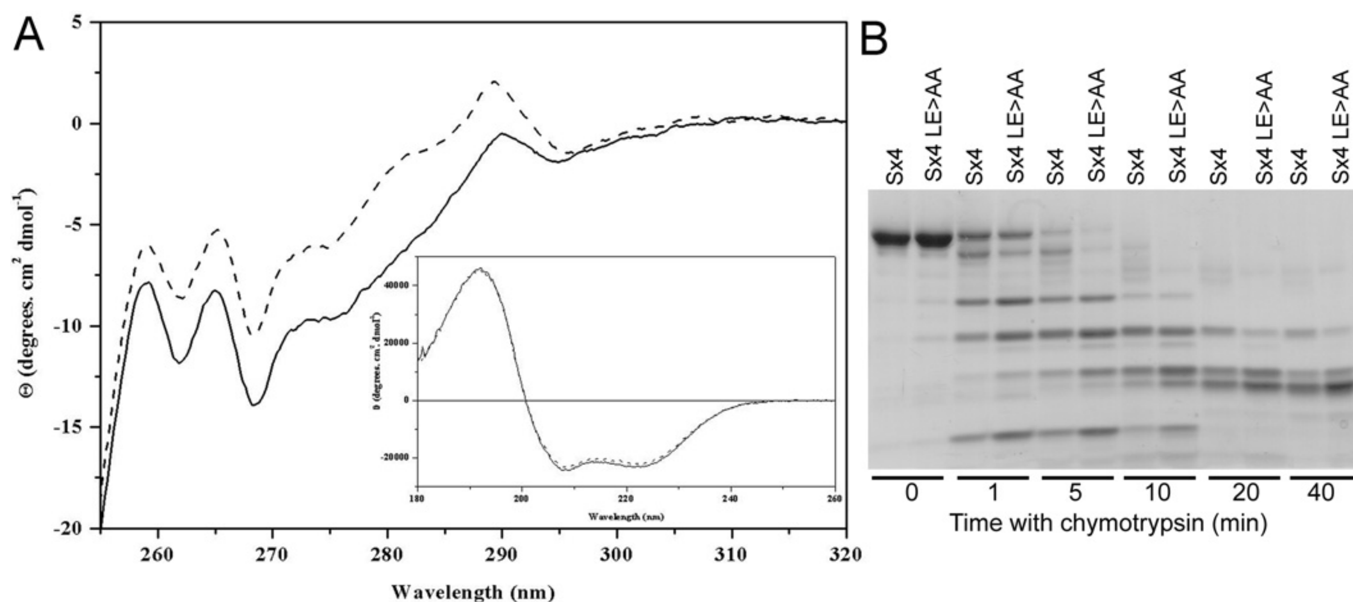

Figure 1 Analysis of the $Sx4_{L173A,E174A}$ mutant

(**A**) Near-UV CD spectra (250–320 nm) were recorded for the cytosolic domains (generated by thrombin cleavage of GST-fusion proteins) of wild-type Sx4 (broken line) and $Sx4_{L173A,E174A}$ (solid line). Proteins were diluted to 1 mg/ml in 50 mM KH_2PO_4/K_2HPO_4 (pH 7.4). Spectra are averaged from four scans. Far-UV CD spectra (insert) were recorded from 180 to 260 nm using proteins diluted to 0.3 mg/ml. Spectra are averaged from eight scans. (**B**) Recombinant wild-type Sx4 or $Sx4_{L173A,E174A}$ (Sx4 LE > AA) were incubated with chymotrypsin (1:100, w/w). Samples were taken and immediately boiled in an equal volume of 2× SDS/PAGE sample buffer at the time points indicated before analysis by SDS/PAGE (12 % gels) followed by Coomassie Blue staining. The gel is representative of three similar experiments.

using *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide chemistry. Following antibody immobilization, residual binding sites on the sensorchip were blocked using 1 M ethanolamine. All measurements were corrected using a double-referencing correction procedure (subtraction of reference flow cell and buffer injections). Munc18c binding was assessed by injecting a series of increasing protein concentrations until a maximum binding concentration was achieved (40 μl/min).

RESULTS AND DISCUSSION

Creation of an open mutant of Sx4

Mutation of both Leu^{165} and Glu^{166}, within the hinge region between the SNARE motif and the Habc domain of Sx1a, to alanine residues results in a protein that is unable to adopt the closed conformation [4]. We mutated the analogous residues in the cytosolic domain of Sx4 (L173A, E174A) in the context of a GST-fusion protein. Analysis by CD demonstrated that these mutations do not cause any gross conformational changes in secondary structure (Figure 1A; the small differences in the near-UV spectra between the wild-type protein and the mutant protein probably reflect localized changes in the environments of aromatic amino acids in the $Sx4_{L173A,E174A}$ mutant). This is consistent with the introduced mutations affecting only the hinge region of Sx4, and not perturbing the structure of either the SNARE domain or the Habc domain, as has been reported for the Sx1a open mutant [4]. To characterize further the $Sx4_{L173A,E174A}$ mutant, we compared its sensitivity to chymotrypsin digestion with that of its wild-type counterpart. The Sx1a-open mutant is more sensitive to proteolysis [22], and Figure 1(B) shows that the $Sx4_{L173A,E174A}$ mutant was also substantially more sensitive to digestion, consistent with it predominantly adopting an open conformation. Through analogy with the Sx1a-open mutant, and the data presented in Figure 1, this mutant is referred to as Sx4-open.

Analysis of Munc18c binding to Syntaxin4

To study the interaction(s) between Sx4 and Munc18c, we first examined the ability of the cytosolic domains of wild-type Sx4, Sx4-open and a further mutant of Sx4 lacking the N-terminal 36 amino acids (Sx4-NΔ) to bind Munc18c. Both wild-type Sx4 and Sx4-NΔ efficiently capture Munc18c in these experiments, whereas Sx4-open exhibits a severely diminished capacity to do so (Figure 2). The observation that Sx4-NΔ binds Munc18c was unexpected, as this mutant lacks the N-terminal region of the syntaxin that has been shown, through crystallographic studies, to interact with Munc18c via the pocket mode of binding [11,12]. Sx4-open contains this N-terminal region and it was therefore also surprising that this mutant displayed a drastically reduced ability to bind Munc18c compared with the full-length cytosolic domain (Figure 2).

We considered the possibility that the bulky GST moiety at the N-terminus of the Sx4 constructs used in Figure 2 was interfering with the pocket mode of binding, and generated the same collection of Sx4 constructs, tagged at the C-terminus. The ability of these to bind Munc18c was investigated as described for the N-terminally tagged constructs presented in Figure 2 (Figure 3). Using the C-terminally tagged constructs, deletion of the N-terminal 36 amino acid residues of Sx4 drastically reduced the ability of Sx4 to capture Munc18c.

The crystal structure of the pocket mode of binding between the N-terminal peptide of Sx4 and Munc18c identifies Leu^8 of Sx4 as making a crucial contact with a hydrophobic pocket in domain 1 of Munc18c [11]. We therefore engineered the L8K mutation, shown previously to reduce Munc18c binding to Sx4 [12], into the C-terminally tagged cytosolic domain of Sx4. Figure 4 shows that

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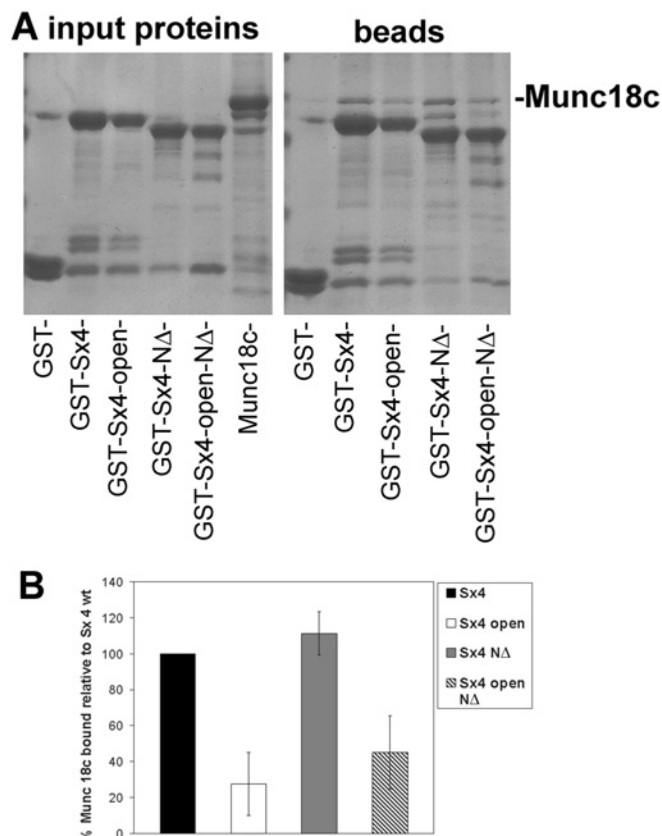

Figure 2 Munc18c binding to N-terminally tagged Sx4

(**A**) A 5 μg sample of GST, GST–Sx4, GST–Sx4-open, GST–Sx4-NΔ or GST–Sx4-open-NΔ was incubated with 10 μl of glutathione–Sepharose for 1 h, and the beads were washed extensively to remove unbound protein. Then, 15 μg of Munc18c was added and incubated overnight at 4 °C with end-over-end rotation. Beads were then washed extensively, and bound proteins were eluted into SDS/PAGE sample buffer. Left-hand panel: purified proteins used in the experiment (input). Right-hand panel: proteins eluted from beads bound to the Sx4 species indicated (beads). The gels are representative of four experiments of this type. (**B**) Quantification of these data, normalized to the amount of Munc18c bound to wild-type (wt) Sx4, expressed as means ± S.D.

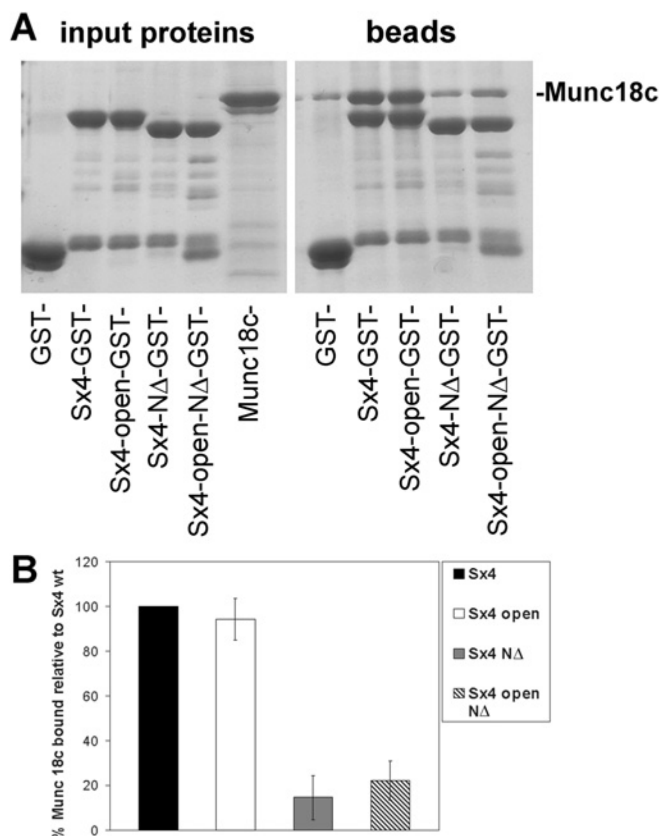

Figure 3 Munc18c binding to C-terminally tagged Sx4

(**A**) Binding of Munc18c to the same set of Sx4 species described in Figure 2 was examined, this time in the context of Sx4 tagged at the C-terminus with GST. Binding was performed exactly as outlined in Figure 2. The gels are representative of an experiment repeated three times. (**B**) Quantification of these data, normalized to the amount of Munc18c bound to wild-type (wt) Sx4, expressed as means ± S.D.

the L8K mutation significantly reduced Munc18c binding to both wild-type Sx4 and Sx4-open, indicating that these proteins both utilize the pocket mode of binding to capture Munc18c. The data presented in Figures 2–4 suggest that the presence of a GST tag at the N-terminus of Sx4 prevents the pocket mode of interaction with Munc18c, perhaps by steric hindrance.

Importantly, the observation that Sx4-open does not bind Munc18c when tagged at the N-terminus indicates that GST–Sx4 and GST–Sx4-NΔ reveal an interaction with Munc18c that is independent of the pocket mode of binding. This interaction is blocked by the N-terminal GST tag in the case of GST–Sx4 and by both the tag and the deletion of residues 1–36 in the case of GST–Sx4-NΔ. The most obvious hypothesis is that this interaction is akin to that reported for Munc18a binding to the closed conformation of Sx1a [5]. Consistent with this, both the GST–Sx4-open and the GST–Sx4-open-NΔ mutants exhibit significantly reduced abilities to bind Munc18c (Figure 2).

When the GST tag is present at the C-terminus of the cytosolic domain of Sx4, the N-terminus is available to contribute to Munc18c binding. The Sx4-open mutant in this context binds Munc18c effectively, because its N-terminus is unhindered and available to use the pocket mode of binding to capture Munc18c (Figure 3). Deletion of the N-terminal 36 residues (Figure 3) or the introduction of the L8K mutation (Figure 4) from this mutant inhibits binding of Munc18c as the pocket mode of binding is now abolished. Hence, we propose that, when tagged at the C-terminus, Sx4 is capable of binding via the pocket mode. Furthermore, the data presented in Figures 3 and 4 indicate that the C-terminally tagged constructs bind predominantly through the pocket mode of binding since its abolition drastically reduces the binding of Sx4–GST to Munc18c (Sx4-NΔ–GST and Sx4-L8K–GST). This suggests that, when tagged with GST at the C-terminus, the cytosolic domain of Sx4 either predominantly adopts an open conformation or is capable of making the closed to open transition effectively.

Figure 1 shows that introduction of two point mutations (L173A and E174A) opens the structure of GST–Sx4 and increases its sensitivity to protease digestion with chymotrypsin. To test the hypothesis that C-terminally tagged Sx4 (Sx4–GST) exists in a predominantly open conformation, we compared its sensitivity to chymotrypsin with that of Sx4 tagged at the N-terminus (GST–Sx4) and the corresponding constitutively open mutants (GST–Sx4-open and Sx4-open–GST). Figure 5 demonstrates that Sx4–GST displays the same sensitivity to chymotrypsin as its corresponding open mutant (compare lanes 7 and 8). In contrast, GST–Sx4 is less sensitive to chymotrypsin than its corresponding open mutant (compare lanes 3 and 4). These data suggest that Sx4–GST is found predominantly in an open conformation in contrast with GST–Sx4.

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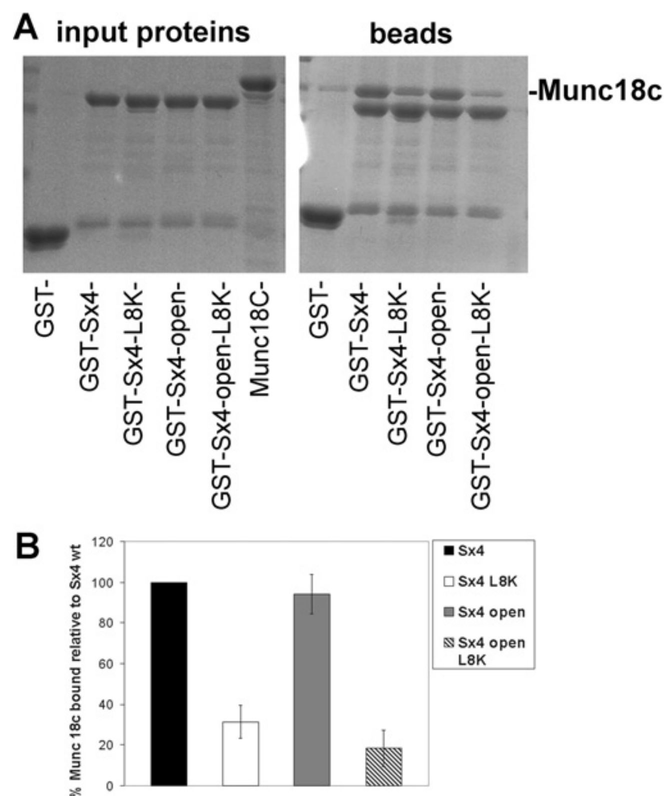

Figure 4 Munc18c binding to the Sx4-L8K mutant

(**A**) Binding of Munc18c to Sx4, Sx4-L8K, Sx4-open or Sx4-open-L8K (in the context of Sx4 tagged at the C-terminus with GST) was examined exactly as outlined in Figure 2. The gels are representative of an experiment repeated three times. (**B**) Quantification of these data, normalized to the amount of Munc18c bound to wild-type (wt) Sx4, expressed as means ± S.D.

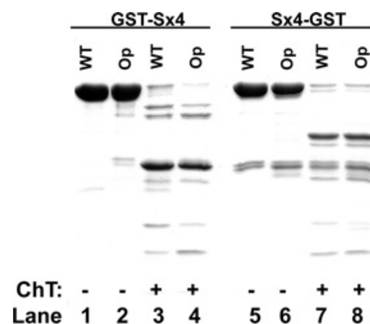

Figure 5 Protease sensitivity of differentially tagged Sx4

Recombinant wild-type Sx4 (WT) or Sx4-open (Op) tagged at either the N-terminus (GST–Sx4, GST–Sx4-open) or the C-terminus (Sx4–GST, Sx4-open–GST) were incubated with or without chymotrypsin (ChT; 1:100, w/w) for 20 min. Samples were then boiled in an equal volume of 2× SDS/PAGE sample buffer before analysis by SDS/PAGE (12 % gels) and Coomassie Blue staining. Gels are representative of three similar experiments.

Our finding that binding of Munc18c to C-terminally GST-tagged Sx4 occurs through the pocket mode of binding utilizing the N-terminal peptide of Sx4, and that the binding of Munc18c to N-terminally GST-tagged Sx4 occurs through a second mode of binding, independent of the pocket mode of binding, perhaps akin to the binding of Munc18a to the closed conformation of Sx1a, afforded the opportunity to measure the affinities of these two interactions in isolation. Using surface plasmon resonance, we found that Sx4–GST has an apparent binding constant (K_d) of ~28 nM, whereas the GST–Sx4 had a K_d of ~254 nM. From these binding data, we propose that the interaction between the N-terminal peptide of Sx4 and Munc18c is a high-affinity interaction, as reported previously [12], whereas the second, previously uncharacterized, interaction is approx. 10-fold weaker. It is noteworthy that a recent study of *in vivo* Sx4–Munc18c interactions studied using fluorescence resonance energy transfer also concluded that regions of Sx4 outwith the N-terminus contribute to Munc18c binding [23]. However, the presence of a CFP (cyan fluorescent protein) moiety at the N-terminus of Sx4 in this previous study may complicate analysis.

Formulation of a unifying hypothesis describing the action of SM proteins has been severely hampered by observations suggesting that different members of this family bind their cognate syntaxins via strikingly different mechanisms [2]. Previous studies, however, have shown that an increasing number of SM protein–syntaxin pairs use multiple modes of binding [15,17–19]. In the present study. we have shown that Sx4 and Munc18c also interact using multiple modes of binding with different affinities, allowing the field to move towards understanding the conserved mechanisms by which SM proteins control membrane fusion. A further important point arising from our study is that the positioning of affinity tags can significantly alter the binding profile of a syntaxin. This could explain why some interactions between SM proteins and syntaxins have been hitherto overlooked.

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