Definition of a minimal munc18c domain that interacts with syntaxin 4

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munc18c is a critical protein involved in trafficking events associated with syntaxin 4 and which also mediates inhibitory effects on vesicle docking and/or fusion. To investigate the domains of munc18c responsible for its interaction with syntaxin 4, fragments of munc18c were generated and their interaction with syntaxin 4 examined *in vivo* by the yeast two-hybrid assay. *In vitro* protein–protein interaction studies were then used to confirm that the interaction between the proteins was direct. Full-length munc18c^{1–592}, munc18c^{1–139} and munc18c^{1–225}, but not munc18c^{226–592}, munc18c^{1–100}, munc18c^{43–139} or munc18c^{66–139}, interacted with the cytoplasmic portion of syntaxin 4, Stx4^{2–273}, as assessed by yeast two-hybrid assay of growth on nutritionally deficient media and by β -galactosidase reporter induction. The

N-terminal predicted helix-a-helix-b-helix-c region of syntaxin 4, Stx4^{29–157}, failed to interact with full-length munc18c^{1–592}, indicating that a larger portion of syntaxin 4 is necessary for the interaction. The yeast two-hybrid results were confirmed by protein–protein interaction studies between Stx4^{2–273} and glutathione S-transferase fusion proteins of munc18c. Full-length munc18c^{1–592}, munc18c^{1–139} and munc18c^{1–225} interacted with Stx4^{2–273} whereas munc18c^{1–100} did not, consistent with the yeast two-hybrid data. These data thus identify a region of munc18c between residues 1 and 139 as a minimal domain for its interaction with syntaxin 4.

Key words: GLUT4, Sec1, SNARE protein, vesicle trafficking.

INTRODUCTION

Transport through both exocytic and intracellular vesicular pathways is mediated by specific vesicle- and target-membrane SNAREs (soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptors), which are highly conserved from yeast to humans [1,2]. These proteins mediate docking and/or fusion by the formation of a SNARE complex between the respective vesicle- and target-membrane SNAREs ([3], see [4] for review). The minimal machinery for this SNARE complex was found to consist of VAMP (vesicle-associated membrane protein) homologue, a vesicle v-SNARE present on the surface of the vesicle, that interacts with syntaxin and SNAP25 (synaptosome-associated protein of 25 kDa) homologues, two cognate receptor t-SNAREs on the target membrane, in a highly specific and stable manner to enable docking and/or fusion. The specificity of fusion was initially believed to be controlled by the interaction of the specific SNARE proteins involved and cellular compartmentalization [5,6]. However, it recently became apparent that non-cognate SNARE proteins could form complexes *in vitro* and that the properties of these complexes were similar regardless of whether the SNARE proteins were closely or distantly related [7]. Therefore the specificity of membrane fusion must be controlled in part by additional events. These events could be mediated through regulatory proteins such as Rab, munc18, SNIP (SNAP25-interacting protein) and synip (syntaxin 4-interacting protein) homologues recently reported to affect the rate of vesicle fusion ([8–16], see [4] for review).

munc18 is a syntaxin-binding protein and is the mammalian homologue of the yeast Sec1 and *Caenorhabditis elegans* Unc18 proteins. These proteins have a critical role in vesicle docking, fusion and/or exocytosis, suggested by the findings that null or temperature-sensitive mutations in yeast or *Drosophila* homologues markedly inhibit vesicle exocytosis [10–13]. Equally, a second, separate, dampening effect on vesicle fusion is clear from studies showing that overexpression of munc homologues inhibits

exocytosis [14–16]. Consistent with these data, studies of *Drosophila melanogaster* expressing different single-point mutations of its munc18 homologue, ROP, identified both loss-of-function and gain-of-function mutations without reaching a clear consensus for domain structure [13]. Such studies suggest separate regulatory roles for munc18 in the vesicle docking and/or fusion process. Understanding the nature of munc18 interactions with its binding partners is critical in assessing these functions.

Currently two families of proteins have been identified that interact with munc18. MINTs were identified by yeast two-hybrid screen as munc-interacting proteins, and appear to play a role in the assembly of proteins involved in vesicle exocytosis and synaptic junctions [17]. Syntaxins also bind specific munc18 isoforms and this binding prevents the interaction of syntaxin with SNAP25/23 and VAMP, and the formation of SNARE complexes required for membrane fusion [18–20].

Thus far three mammalian munc18 isoforms have been identified, munc18a, b and c [18,21,22]. munc18a and b were found to interact with syntaxins 1A, 2 and 3 but not 4 in both protein–protein-interaction and yeast two-hybrid-system studies [21]. The third isoform, munc18c, was found to bind to syntaxin 2 and 4, and to a lesser extent to syntaxin 1A [22]. munc18c has been examined primarily in the context of the trafficking of the insulin-responsive glucose transporter, GLUT4, to the plasma membrane in fat and muscle [15,16,20]. Overexpression of munc18c in 3T3-L1 cells inhibited insulin-stimulated glucose transport whereas overexpression of munc18b was without effect [15]. Consistent with these findings, overexpression of munc18c inhibited GLUT4, but not GLUT1, translocation to the plasma membrane [15,16], whereas overexpression of munc18b was without effect on GLUT translocation. These studies imply a potential regulatory role for munc18c in GLUT4 translocation and glucose transport.

The domain of munc18 involved in its interaction with syntaxin is of interest since it represents a potential regulatory point in vesicle docking and/or fusion. However it was found previously

Abbreviations used: SNARE, soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor; VAMP, vesicle-associated membrane protein; SNAP25, synaptosome-associated protein of 25 kDa; HaHbHc domain, helix-a-helix-b-helix-c domain; GST, glutathione S-transferase; GSK3, glycogen synthase kinase-3.

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that even a small modification of munc18a eliminated its binding to syntaxin 1A [21]. Specifically, deletion of a 16-residue fragment from the N-terminus of munc18a, or a 25-residue fragment from the C-terminus, abolished its interaction with syntaxin 1A. The conclusion from these studies was that full-length munc18a was essential for its interaction with syntaxin 1A [21].

In the present study, a minimal interacting domain for munc18c that binds to syntaxin 4 has been defined using both protein–protein interactions and the yeast two-hybrid system. In contrast to previous findings for the interaction of munc18a with syntaxin 1A [21], we found that the N-terminal 139 amino acids of munc18c were sufficient for interaction with syntaxin 4. These studies thus indicate that there may be differences in the detail of munc-isoform interactions. These differences may contribute to the specificity of vesicle docking and/or fusion.

MATERIALS AND METHODS

Constructs

Full-length mouse munc18c (residues 1–592, GenBank accession number U19521; a gift from Professor D. James, University of Queensland, St. Lucia, QLD, Australia) was amplified by PCR using primers N5603 and N5604 (Table 1) and subcloned into yeast two-hybrid vectors pGAD424 [23] and pAS2 (Clontech, Palo Alto, CA, U.S.A.). Deletion mutants of these constructs based on a unique *StuI* site in munc18c yielded constructs spanning residues 1–225 and 226–592 (munc18c^{1–225} and munc18c^{226–592}). Deletion constructs of munc18c spanning residues 1–139, 1–100, 66–139 and 43–139 (munc18c^{1–139}, munc18c^{1–100}, munc18c^{66–139} and munc18c^{43–139}) were generated by PCR using the oligonucleotide primers listed in Table 1 and cloned into pAS2 and pGEX-KG. The complete cytoplasmic portion of syntaxin 4 (amino acids 2–273, Stx4^{2–273}) was cloned as previously reported [24]. A fragment of syntaxin 4 corresponding to amino acids 29–157 (Stx4^{29–157}), corresponding to the analogous helix-a-helix-b-helix-c (HaHbHc) domain described for syntaxin 1A [25,26], was generated by PCR using primers N5868 and N5869, characterized and cloned into pGAD424 (the prey vector), pAS2 (the bait vector) and pGEX-KG. pAS2:glycogen synthase kinase-3 (pAS2:GSK3; a gift

from Peter Roach, Indiana University, Indianapolis, IN, U.S.A.) was used as a negative control.

Yeast two-hybrid assay

Co-transformations of yeast strain CG1945 (*MATa*, *ura3-52*, *his3-200*, *leu2-3*, *112 lys2-801*, *trp1-901*, *ade2-101*, *gal4-542*, *gal80-538*, *URA3:(GAL4 17-mers)₃-CYC1-lacZ*, *LYS2:GAL1-HIS3*, *cyh'2*) with Stx4^{2–273}, Stx4^{29–157} or pAS2:GSK3 (negative control) together with the munc18c constructs were performed as described previously [27,28] and plated on media selective for colonies able to synthesize leucine and tryptophan. Positive interactions were selected by the ability of colonies to grow on plates deficient in tryptophan, leucine and histidine (Trp[–]Leu[–]His[–]).

Interactors were assessed further by the measurement of β -galactosidase activity on filter lifts of the clones following growth at 30 °C for 2 days. Interaction strength was then assessed quantitatively in liquid culture by assaying the specific activity of β -galactosidase generated by the inducible *lacZ* reporter gene. Briefly, exponentially growing cultures were harvested by centrifugation, lysed using glass beads in lysis buffer (100 mM Tris, pH 8.0, 1 mM dithiothreitol, 20 % glycerol and 1 mM PMSF) and cell debris removed by centrifugation at 12000 *g* for 5 min. β -Galactosidase activity of the lysates was measured at 420 nm as the rate of hydrolysis of the substrate *o*-nitrophenyl β -D-galactopyranoside [29] as a function of protein concentration.

Glutathione S-transferase (GST) fusion proteins and protein purification

A GST fusion protein consisting of Stx4^{2–273} was produced as described previously [24] from a pGEX construct in *Escherichia coli* strain DH5 α . GST fusion proteins consisting of munc18c^{1–100}, munc18c^{1–139}, munc18c^{1–225} and munc18c^{1–592} were produced from pGEX constructs in *E. coli* strain DH1 containing a GroEL plasmid under chloramphenicol selection and transformed with the pGEX-munc constructs. Cells were selected on ampicillin- and chloramphenicol-containing agar plates.

GST fusion proteins were purified on glutathione–Sephacryl (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) using standard procedures [29]. Briefly, *E. coli* transformants were grown in YT media with 100 μ g/ml ampicillin. Protein production was induced with 0.1 mM isopropyl β -D-thiogalactoside for 2 h. Protein was extracted from the cells by sonication, lysis in 1 % Triton X-100 in PBS, pH 7.4, and centrifugation at 12000 *g* for 10 min. Protein in the supernatant was purified on glutathione–Sephacryl. GST-munc18c fusion proteins were used immobilized on glutathione–Sephacryl. Stx4^{2–273} was used in free form after cleavage of GST with 100 units of thrombin while the protein was attached to the glutathione–Sephacryl resin. Stx4^{2–273} was then dialysed against Tris-buffered saline, pH 7.4, for use.

In vitro protein–protein interaction assessment

GST fusion proteins of munc18c^{1–100}, munc18c^{1–139}, munc18c^{1–225} and munc18c^{1–592}, immobilized on glutathione–Sephacryl, were matched approximately for amount (1–2 μ g), and incubated with 10 μ g of Stx4^{2–273} in 300 μ l of PBS containing 1 % Triton X-100 for 60 min at 4 °C. The glutathione–Sephacryl was then washed five times in the same buffer and syntaxin 4 bound to the GST-munc18c proteins analysed after SDS/PAGE and Western transfer by probing transfer filters with a syntaxin 4 antibody (rabbit polyclonal antibody raised against GST-syntaxin 4) using

Table 1 Oligonucleotide primers used for the PCR of munc18c and syntaxin 4 constructs

All sequences are shown 5' → 3'.

Construct	Strand	Primer	Sequence
munc18c	+	N5603	GCGAATTCATGGCGCCGCGGTATCGGAG
		N5604	AGCGGGATCCTTACTCATCCTTAAGGAAAC
	+	N5893	TAGACTGGATCCATGGCGCCGCGGTATCGGAG
		N5900	TAGACTGAATTCGTCGACTTAGGAAATGTTTATTCCTTAC
	+	N5893	As above
		N5963	TAGACTGAATTCGTCGACTTACTCAGATTTACTCCAAATC
	+	N6046	TAGACTGGATCCAAACTTTTGTGTCATCGTGC
		N5900	As above
	+	N6047	TAGACTGGATCCTATAAGCGTGAACCTGTC
		N5900	As above
Stx4	+	N5868	ACGCTAGGATCCCCGGGCACGGCAGCGCTG
		N5869	AGCTGAGAATTCGTCGACTTACCGAATCCGCTCCACGTTT

chemiluminescence detection (Pierce Super Signal®, Pierce, Rockford, IL, U.S.A.). The proteins were also analysed following SDS/PAGE by Coomassie Brilliant Blue staining.

RESULTS

Yeast two-hybrid assessment of munc18c–syntaxin 4 interactions

Direct protein–protein interaction studies with munc18c have been hampered by the low solubility of the protein *in vitro*. The yeast two-hybrid system provides an alternative approach that allows *in vivo* assessment. In the present study, the yeast two-hybrid system was used to study the interaction of munc18c with the full cytoplasmic portion of syntaxin 4, Stx4^{2–273}, as well as the N-terminal three-helical domain of syntaxin 4 (Stx4^{29–157}). When munc18c and syntaxin 4 were ligated into the bait vector, pAS2, and prey vector, pGAD424, respectively, a productive interaction was demonstrated by growth on plates nutritionally deficient in Trp, Leu and His (Figure 1A) and by β -galactosidase production (Figure 1B). In contrast, when the constructs were cloned into the vectors in the opposite format, pAS2:syntaxin 4 and pGAD424:munc18c, only a weak interaction was observed (results not shown). Figure 1(A) shows that growth on Trp[−]Leu[−]His[−] plates was observed with media plated with yeast expressing Stx4^{2–273} and full-length munc18c^{1–592}, munc18c^{1–139} or munc18c^{1–225}, but not munc18c^{226–592}, munc18c^{1–100} or munc18c^{43–139} (Figure 1A). No growth was observed when pGAD424:syntaxin 4 was tested against pAS2:GSK3 as a negative control. Like the growth assay, the β -galactosidase-reporter-assay data indicate that Stx4^{2–273} interacted with full-length munc18c^{1–592}, munc18c^{1–139} and munc18c^{1–225} but not with munc18c^{226–592}, munc18c^{1–100}, munc18c^{43–139} or munc18c^{66–139} (Figure 1B). Stx4^{29–157} did not interact with munc18c^{1–592} or any of the shorter munc18c fragments, as assessed by filter assay of β -galactosidase reporter activity (Figure 1B).

(A)

(B)

pAS2 Munc	1-592	1-225	1-139	1-100	226-592	43-139	66-139
pGAD Stx4	+	+	+	−	−	−	−
pGAD Stx4 ^{29–157}	−	NA	−	NA	NA	−	−

Figure 1 Interactions between syntaxin 4 and munc18c constructs

The full-length cytoplasmic portion of syntaxin 4, Stx4^{2–273}, or Stx4^{29–157} encompassing the N-terminal HaHbHc region of syntaxin 4, were ligated into the pGAD424 vector as described in the Materials and methods section. munc18c constructs were cloned into the pAS2 vector. Yeast strain CG1945 was then transformed sequentially with both vector constructs, grown on media lacking Trp and Leu and co-transformants assayed for their ability to grow on media lacking Trp, Leu and His (A: pGAD424:Stx4^{2–273} with pAS2:munc18c constructs) or to produce β -galactosidase (B: both pGAD424:Stx4^{2–273} and pGAD424:Stx4^{29–157} with pAS2:munc18c constructs). +, β -galactosidase production; −, indicates no detectable β -galactosidase activity; NA, not assessed.

Figure 2 Quantitative β -galactosidase activities of syntaxin 4 and munc18c co-transformants

Yeast strain CG1945 co-transformed with the cytoplasmic portion of syntaxin 4, pGAD424:Stx4^{2–273} and pAS2:munc18c constructs were grown in liquid media lacking Trp and Leu at 30 °C. Cells were harvested and lysed by homogenization with glass beads and β -galactosidase activity in the supernatant was determined using *o*-nitrophenyl β -D-galactopyranoside and by measuring absorbance at 420 nm after correction for protein as described in the Materials and methods section.

Assay of β -galactosidase activity in yeast extracts

The β -galactosidase activity of yeast-extract supernatants generated from the interaction of pGAD424:syntaxin 4 with pAS2:munc18c^{1–592} and fragments was quantified (Figure 2). β -Galactosidase activity generated by cells co-transformed with pGAD424:syntaxin 4 and pAS2:munc18c^{1–592} exhibited approxi-

Figure 3 *In vitro* protein-protein-interaction studies between munc18c fusion proteins and syntaxin 4

GST fusion proteins of munc18c¹⁻¹⁰⁰, munc18c¹⁻¹³⁹, munc18c¹⁻²²⁵ and full-length munc18c¹⁻⁵⁹², immobilized on glutathione-Sepharose, were incubated with 10 μ g of syntaxin 4 (STX) for 60 min at 4 °C. The glutathione-Sepharose was then washed and bound syntaxin 4 was analysed by SDS/PAGE using (A) Coomassie Brilliant Blue staining, or (B) Western transfer by syntaxin 4 antibody binding, as described in the Materials and methods section. Results are representative of three separate studies.

mately twice the activity as that generated by cells co-transformed with pGAD424:syntaxin 4 and pAS2:munc18c¹⁻¹³⁹, while cells co-transformed with pAS2:munc18c¹⁻²²⁵ showed less than a third of the specific activity compared with munc18c¹⁻⁵⁹² (Figure 2). The other two constructs, pAS2:munc18c¹⁻¹⁰⁰ and pAS2:munc18c²²⁶⁻⁵⁹², did not show any activity (Figure 2), consistent with both the growth and filter assays (Figure 1).

In vitro munc18c-syntaxin 4 interaction studies

The binding of syntaxin 4 to the various munc18c fragments was examined to confirm the results of the yeast two-hybrid studies and to demonstrate that the proteins interacted directly (Figure 3). Immobilized GST-munc18c fusion proteins (1–2 μ g) were incubated with excess (10 μ g) syntaxin 4, washed and the interactions analysed by SDS/PAGE, Coomassie Brilliant Blue staining and Western blotting. Each interaction was matched approximately for munc18c protein immobilized on glutathione-Sepharose, as assessed by Coomassie Brilliant Blue staining of SDS/PAGE gels, although in the experiment shown (Figure 3A), the amount of munc18c¹⁻¹³⁹ incubated with syntaxin 4 was slightly higher than for the other proteins. No Coomassie Brilliant Blue-stained band was detected at the expected molecular mass for syntaxin 4, 35 kDa, for any of the munc18c proteins interacted with syntaxin 4 (Figure 3A). However, Western-blot analysis detected syntaxin 4 in interactions with GST-fused proteins of munc18c¹⁻⁵⁹², munc18c¹⁻¹³⁹ and munc18c¹⁻²²⁵, but not

munc18c¹⁻¹⁰⁰. Binding to full-length munc18c, munc18c¹⁻⁵⁹², appeared greater than that to the shorter fragments, consistent with the results from the yeast two-hybrid interactions. The amount of syntaxin 4 bound to the GST-munc constructs was sub-stoichiometric and only of the order of 0.03 μ g. This suggests that the interaction is of low affinity or that a proportion of the syntaxin and/or munc is in a conformation that fails to bind. In support of the latter possibility, syntaxin 1A has been shown to exist in two different conformations when bound, and in a third in isolation [26,30]. The conditions required for a structural transition of syntaxin 4 from an unbound state to a munc18c-bound state are not yet understood. Alternatively, recombinant munc18c has a tendency to aggregate (results not shown) and this may mask the binding site to some extent.

DISCUSSION

The present study focused on the interactions between munc18c and syntaxin 4, a plasma-membrane t-SNARE shown to be important in a number of vesicle-trafficking events associated with the plasma membrane, including translocation of the insulin-responsive glucose transporter, GLUT4, to the plasma membrane, and glucose transport [16,20,30–33]. In contrast to previous findings for the interaction of munc18a with syntaxin 1 [21], it was found that full-length munc18c was not required for binding to syntaxin 4. Rather, the N-terminal fragments munc18c¹⁻²²⁵ and munc18c¹⁻¹³⁹ were able to bind to syntaxin 4, whereas the C-terminal fragment, munc18c²²⁶⁻⁵⁹², showed no binding ability at all. Further, deletion of the N-terminal 42 amino acids, in munc18c⁴³⁻¹³⁹, resulted in loss of interacting activity.

These findings are consistent with previous data that examined binding of fragments of the yeast munc18 homologue Sly1p to its cognate syntaxin homologue Sed5p [34]. Sly1p¹⁻²⁹⁴ bound to Sed5p, although not to the same extent as full-length Sly1p, whereas a C-terminal Sly1p fragment, Sly1p³⁴⁰⁻⁶⁶⁶, and shorter N-terminal fragments, Sly1p¹⁻¹⁹⁷ and Sly1p¹⁻¹⁰⁷, failed to interact. Interestingly, in the present studies, the smallest munc18c fragment that interacted with syntaxin 4, munc18c¹⁻¹³⁹, was shorter than that for Sly1p. The reasons for this are not clear. However, it is possible that shortening the 1–139 structure to 1–100 in munc18c or 1–107 in Sly1p destabilizes the structure, since the crystal structure of the munc18a-syntaxin 1A complex [30] indicates there to be no contacts with syntaxin in this region of munc. This may also explain the lack of binding of the Sly1p¹⁻¹⁹⁷ construct [34]. Alternatively it is possible that the molecular details of the interactions of each homologue differ to some extent. The recent structural description of the syntaxin 1A-munc18a complex identified a major surface of contact on munc18a between residues 38 and 71 and further identified a domain structure for munc18a [30]. Domain 1, consisting of residues 4–134 of munc18a, was not dissimilar to our minimal binding construct, munc18c¹⁻¹³⁹, and was the major point of contact with syntaxin 1A [30]. Our construct, determined empirically, was two residues longer than munc18a domain 1, terminating in the same inter-domain loop between β 5 and β 6 of munc18a [30] and hence likely to adopt a stable structure.

Another potential interacting domain was identified in previous studies to reside within a peptide identified by homology between different munc18 species, namely squid Sec1⁴⁶⁵⁻⁴⁸⁹. This peptide was found to inhibit binding of s-secl1 to s-syntaxin and to block neurotransmission when microinjected [35]. The homologous region of munc18c was also shown recently to inhibit binding of full-length munc18c to syntaxin 4 and to inhibit GLUT4 translocation [36]. However, this peptide lies in the

region of munc18c, which was found here not to interact with syntaxin 4, and of Sly1p, which did not interact with Sed5p [34]. Further, in the munc18a–syntaxin 1A crystal complex, the peptide includes a loop reported to be well separated from the syntaxin 1A-binding site [30]. It is possible therefore that the peptide binds syntaxin in a different conformation to that in the crystal complex and to the studies reported here. If this is the case, it is possible that this region may contribute to the enhanced binding of the full-length munc18c compared with the N-terminal peptides.

A further aspect of the syntaxin 4–munc18c interaction is the region of syntaxin 4 that interacts with munc18c. The yeast two-hybrid studies described here were performed with the full cytoplasmic portion of syntaxin 4 (excluding the initiating methionine), Stx4^{2–273}, and a fragment of syntaxin 4, Stx4^{29–157}, corresponding to the analogous HaHbHc domain described from syntaxin 1A. This fragment encompasses the major structural region of syntaxin outside the SNARE motif and in syntaxin 1A has been reported to have apparent regulatory function [25]. In yeast it has been shown previously that the syntaxin homologue Sed5p N-terminal helix (1–78) was sufficient for binding to Sly1p [37]. Further, an unrelated protein, munc13, has been shown to interact with the N-terminal portion of syntaxin 1, Stx1^{1–79}, supporting the notion that this region of the protein is involved in protein–protein interactions [38]. However no interaction was detected for the HaHbHc fragment of syntaxin 4 used in the present study. munc18c binding required full-length syntaxin 4. These data are consistent with previous studies that demonstrated a minimal requirement of residues 1–240 of syntaxin 1A for binding to munc18a [39]. The recent description of the syntaxin 1A–munc18a complex demonstrated contact between domain 1 of munc18a (residues 4–135) and a number of residues within the HaHbHc domain and the SNARE motif through to residue 243 of syntaxin 1A [30]. Other studies have suggested the N-terminal portion of syntaxin 1 to be of importance for munc18 binding [40,41]. These studies demonstrated that deletion of the N-terminal 16 amino acids of syntaxin inhibited its binding to munc18. However, it was also found that deletion of the C-terminus of syntaxin 1 abolished binding [40], consistent with the present studies demonstrating the N-terminal portion of syntaxin 4 was insufficient for munc18c binding. In view of these studies it is of interest that the N-terminus of the yeast syntaxin homologue Sed5p is sufficient for its interaction with Sly1p [37].

The present study demonstrates that the N-terminal portion of munc18c interacts with syntaxin 4 and suggests the requirement of a significant portion of syntaxin 4 (at least, larger than Stx4^{29–157}) for this interaction. This might be expected if the interaction involved syntaxin 4 in a closed conformation, as has recently been demonstrated for syntaxin 1 [26,30,42].

An inhibitory effect of munc18 and its homologues on vesicle fusion has been demonstrated in a number of overexpression studies, interpreted as being due to saturation binding of munc18 to syntaxin homologues, thereby preventing VAMP-homologue binding [15,16,35,43]. However, an essential positive role for munc18 in vesicle trafficking is supported by the findings that null or temperature-sensitive mutations in *C. elegans*, *Saccharomyces cerevisiae* and *D. melanogaster* produce a phenotype in which vesicle exocytosis is markedly inhibited [10–12]. Micro-injection of a peptide located near the C-terminal portion of squid sec1 into squid axons led to the accumulation of docked synaptic vesicles but prevented fusion [35]. Similarly, micro-injection of the analogous munc18c peptide inhibited fusion of GLUT4 vesicles with the plasma membrane and led to their accumulation below the cell surface [36]. It appears therefore

that this peptide region is either not involved in the fusion event, or requires other regions of munc18/sec1 as well. It is possible that the N-terminal portion of munc18 may be important for this function. However, since the N-terminal portion of munc18c clearly binds syntaxin alone, a role in both the inhibition of SNARE-complex formation and the stimulation of fusion is possible. The interaction studies reported here provide information on the domains of munc18c that, put together with the information provided by the syntaxin 1A–munc18a structure determination [30], obviously now needs to be examined in a cellular context. These studies are being pursued at present.

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