Milligram Quantities of Homogeneous Recombinant Full-Length Mouse Munc18c from *Escherichia coli* Cultures

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**Abstract**

Vesicle fusion is an indispensable cellular process required for eukaryotic cargo delivery. The Sec/Munc18 protein Munc18c is essential for insulin-regulated trafficking of glucose transporter4 (GLUT4) vesicles to the cell surface in muscle and adipose tissue. Previously, our biophysical and structural studies have used Munc18c expressed in SF9 insect cells. However, to maximize efficiency, minimize cost and negate any possible effects of post-translational modifications of Munc18c, we investigated the use of *Escherichia coli* as an expression host for Munc18c. We were encouraged by previous reports describing Munc18c production in *E. coli* cultures for use in *in vitro* fusion assay, pulldown assays and immunoprecipitations. Our approach differs from the previously reported method in that it uses a codon-optimized gene, lower temperature expression and autoinduction media. Three N-terminal His-tagged constructs were engineered, two with a tobacco etch virus (TEV) or thrombin protease cleavage site to enable removal of the fusion tag. The optimized protocol generated 1–2 mg of purified Munc18c per L of culture at much reduced cost compared to Munc18c generated using insect cell culture. The purified recombinant Munc18c protein expressed in bacteria was monodisperse, monomeric, and functional. In summary, we developed methods that decrease the cost and time required to generate functional Munc18c compared with previous insect cell protocols, and which generates sufficient purified protein for structural and biophysical studies.

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**Introduction**

Sec/Munc18 (SM) and SNARE proteins are essential for vesicle exocytosis in eukaryotes [1–7]. The assembly of the fusogenic SNARE complex is regulated in part by SM proteins through interaction with their cognate SNARE Syntaxin (Sx), in distinct vesicle transport pathways [3,8–15]. Deletion and over-expression of SM proteins have shown both positive and negative effects in each step of vesicle fusion [3,16–18]. Three of the seven SM isoforms expressed in mammals are involved in exocytosis: Munc18a, Munc18b and Munc18c [19–21]. Munc18a is expressed specifically on neuronal cells and is required for neurotransmission. By contrast, Munc18c is ubiquitously expressed [22] and is required for GLUT4 translocation to the cell surface in adipose/muscle tissues in response to insulin signaling, and is also important in endothelial cell activation [23–27].

While Munc18 proteins are essential components of vesicle fusion their mode of regulating vesicle fusion is poorly understood [4,6,10,17,28]. Thus, Munc18c:Ex complexes have been reported to inhibit SNARE complex formation [12,13,29–32] or permit SNARE complex formation [33–38]. We are interested in unraveling the role of Munc18c in insulin-stimulated membrane fusion [23,27,29,33,35,39–43] and have previously used recombinant Munc18c expressed in baculovirus infected insect cells [44] for our structural and biophysical studies. However, this approach is relatively expensive and time-consuming to generate the milligram quantities required on a regular basis, and we cannot rule out the possibility that the produced protein has unintended post-translational modifications that affect its interactions with partner proteins.

Several groups have reported the production of recombinant Munc18c using an *E. coli* expression system to generate recombinant Munc18c protein for their studies (summarized in Table 1). For example, Munc18c has been cloned into pQE30 (creating an N-terminal His fusion protein) and co-expressed with GroEL in M15 *E. coli* cells for *in vitro* pull-downs and liposome fusion assays [29]. Others have used bacterially expressed Munc18c for specific assays (see Table 1 for a summary). However, using the same methods, we were unable to produce sufficient recombinant full-length Munc18c protein for our structural biology and biophysical studies.

Here we report the large-scale production of purified recombinant Munc18c from a codon optimized full-length synthetic mouse gene. We followed the lead of Brandie et al., 2008, by using a pQE30 vector and co-expressing Munc18c with GroEL/GroES chaperones to assist folding. The optimal expression conditions in *E. coli* BL21 cells include the use of auto-induction media and a very low expression temperature (16°C), which delivers 1 mg of...
Table 1. Reported Munc18c expression and purification.

| Origin                        | Residues   | Construct                          | Expression                        | Purification                  | Yield   | Used for                      | Reference |
|-------------------------------|------------|------------------------------------|-----------------------------------|------------------------------|---------|-------------------------------|-----------|
| Mouse cDNA                    | FL (1-592) | Munc18c- His<sub>6</sub>            | *E. coli* BL21 (DE3)              | IMAC (Ni-NTA)                | NR<sup>*</sup> | PDA or IP                     | [43]      |
| Mouse cDNA                    | FL (1-592) | pET28a-His<sub>6</sub>-Munc18c     | *E. coli* cells                   | IMAC (Ni-NTA)                | NR<sup>*</sup> | PDA or IP                     | [41,49]   |
| NR<sup>*</sup> FL (1-592)      | pQE30-His<sub>6</sub>-Munc18c | Co-expressed with GroEL in *E. coli* M15 cells, Media NR; IPTG induction at 25°C | IMAC (Ni-NTA)                | NR<sup>*</sup> | In-vitro fusion assays,       |           |
| PDA                           |            |                                    |                                   |                              |         |                               | [29,39]   |
| Rat Munc18c                   | FL         | pGEX-KG-Munc18c                    | *E. coli* BL21 (DE3) (RIPL) with IPTG (100 nM) induction at 27°C | Protein was purified using glutathione Sepharose beads for GST-moiety | NR<sup>*</sup> | PDA                           | [40]      |
| Human Munc18c cDNA            | FL (1-592) | pQE-9-His<sub>6</sub>-Munc18c      | Expressed in *E. coli*            | Protein produced in inclusion body was solubilized by 8 M urea and purified on Ni-NTA beads | NR<sup>*</sup> | IP                            | [26]      |
| Mouse Munc18c                 | FL (1-592) | pAc-HLT-B-His<sub>6</sub>-Munc18c or pFast-Bac-His<sub>6</sub>-TEV-Munc18c | Insect SF9 cells                  | IMAC (TALON) followed by SEC | 2–4 mg/L | Purification and Characterization; PDA, SAXS, cross-linking, ITC; in-vitro fusion assays | [33,35,38,44] |
| Mouse (codon optimized synthetic gene) | FL (1-592) | pQE30-His<sub>6</sub>-Munc18c      | Co-expressed with GroEL in *E. coli* BL21 cells, auto-induction (ZYP) media at 16°C | IMAC (PrepEase) followed by SEC and IEC | 1–2 mg/L | Purification and Characterization; ITC, PDA | This work |

NR<sup>*</sup>- Not reported, FL- full-length, PDA- pull down assays; IP- immunoprecipitation; ITC- isothermal titration calorimetry; SAXS- small angle X-ray scattering.
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recombinant purified Munc18c proteins (with different tags) for L. culture. The recombinant protein was folded, stable, monomeric, mono-disperse and functional. By optimizing the expression in E. coli we developed a protocol and constructs to generate the amounts of purified Munc18c required for future biophysical and structural studies.

Materials and Methods

Munc18c constructs

Fig. 1 provides a summary of the constructs used in this work. The wild type Munc18c gene was PCR-amplified from the previously described pAcHLT-B-Munc18c construct [44] and was then sub-cloned into a pGEX vector (GE Healthcare, UK) between BamHI and Smal (New England Biolabs, USA) restriction sites using specific forward (5'-CACCAGGATCCGAGAAGGAGC-3') and reverse primers (5'-GACCAGGGAAGTTATCACTCATGCTTAAAGG-3'). To generate a construct similar to that described by Brandie et al., (2008), the pGEX-Munc18c and pQE30 plasmids were digested with BamHI and Smal (New England Biolabs, USA) and the excised Munc18c gene was inserted into the pQE30 vector, and ligated using T4-ligase (New England Biolabs, USA). This construct is referred to as HMunc18c (Fig. 1), and its sequence was verified as described above.

To mimic the construct used for insect cell expression [44], the pQE30-Munc18c plasmid was modified to produce the cleavable fusion sequence MSPIDPMGHHHHHHGRRASVAG-GLIVPRGSPGLDGTYARGIQASMAAGFG (thrombin recognition site is underlined). To insert this linker in the plasmid, pQE30-Munc18c was digested with EcoRI and BamHI to remove the coding region for the original His6 tag. The coding sequence for the linker (purchased from GenScript, NJ – See Fig. S1B) was also digested with EcoRI and BamHI and then ligated into the digested pQE30-Munc18c plasmid using T4-ligase (New England Biolabs, USA). This construct is referred to as HLMunc18c (His-TEV-Munc18c - Fig. 1), and its sequence was verified as described above.

An untagged Munc18c construct was generated by PCR amplification, using pQE30-HMunc18c as the template and the primers 5'-CATCACCATCACCATCACCATCACCATCACCATCACCATCACCATCACCATCACCATCACCATCACCATCACCATCACCATCAC-3' and 5'-GCCGGATCCGAGAAGGAGC-3' (the coding region for the TEV site is underlined). The PCR product was then digested using BamHI and then circularised using T4-ligase (NEB, USA). This construct is referred to as HLMunc18c (His-Linker-Munc18c - Fig. 1), and its sequence was verified as described above.

Test Expression in E. coli

The plasmid encoding HLMunc18c or HMunc18c was co-transformed with the pREP4-GroESL plasmid [45] into E. coli M15 or BL21 for test expression. Transformation mixtures (50 µL) were plated onto Luria Bertani (LB) agar, and selective pressure

Figure 1. Munc18c and Sx4 constructs. The constructs used in this work or referred to in the text are shown, including the names, fusion tags and molecular mass of each construct. The sequence of the His6 linker for HLMunc18c and HLMunc18c is given in the text. doi:10.1371/journal.pone.0083499.g001
for clones containing both the pQE30-Munc18c and pREP4 plasmids was applied with ampicillin (100 µg/mL) and kanamycin (50 µg/mL). After 24 h, a single colony was used to inoculate 5 mL LB containing both ampicillin and kanamycin. The culture was incubated at 37°C with shaking overnight. For test expression, 1 L of media (LB, terrific broth (TB) media or ZYP-5052 auto-induction [46]) containing ampicillin (100 µg/mL) and kanamycin (50 µg/mL) was inoculated with overnight culture (1 mL). All cell cultures were grown at 37°C to an optical density at 600 nm (OD600) of 0.6. LB and TB cultures were then induced with 1 mM IPTG and incubated at 25°C, 20°C or 16°C whilst auto induction cultures were directly incubated at 25°C, 20°C or 16°C. Cell growth in LB and TB media at 16°C was very slow and these were not continued further. After incubation for 20 to 22 h, cells were harvested by centrifugation ([JLA 8.1 rotor, AVANTI centrifuge (Beckman Coulter, USA), 5000 g, 15 min at 4°C]. Pelleted cells (1 L cell pellets) were resuspended in Tri lyso buffer (25 mM Tris-HCl pH 7.5; 300 mM NaCl, 10% (w/v) glycerol, 1% (w/v) Triton X-100, 5 mM imidazole, 2 mM 2-mercaptoethanol (β-ME), 10 mM MgCl₂) at a ratio of 10 mL per gram of wet pellet. To this mixture, 12,500–14,000 units DNase (Roche) and 100 µL of Bacterial Protease Inhibitor (BioPioneer, Inc., USA) was added, and the cells then lysed by sonication (10 Hz pulses for 30 sec at 30% amplification on an Ultrasonic homogenizer (BioLogics Inc., USA) to a final concentration of 400 µg/mL. After incubation, the lysate was centrifuged at 18,500 g, 30 min at 4°C. After incubation, the cell debris was removed by centrifugation ([JLA 16.25 rotor, AVANTI centrifuge (Beckman Coulter, USA), 13,500 g, 30 min, 4°C].) and the soluble fraction was incubated with 0.5 mL of Co²⁺-affinity beads (TALON beads, Takara Bio Inc, Japan) pre-equilibrated in Tri lysis wash buffer (25 mM Tris-HCl pH 7.5; 300 mM NaCl, 10% (w/v) glycerol, 20 mM imidazole, 2 mM β-ME) per 100 mL lysate for 2 h at 4°C to bind the His₆-tagged Munc18c. After 2 h, the beads were washed in five column volumes of Tri wash buffer and protein was eluted in 1 mL aliquots using Tri elution buffer (Tri wash buffer plus 300 mM imidazole). Samples were analysed by SDS-PAGE gels.

Large-Scale Expression of Munc18c in E. coli

For large-scale protein production, 2–6 L cultures were used. The plasmid vector encoding the codon-optimized Munc18c gene (HMunc18c, HTMunc18c, HLMunc18c or untagged Munc18c) was freshly co-transformed into E. coli BL21 with the pREP4 plasmid encoding the GroEL/ES chaperones, and grown in culture as described above using autoinduction and moving cultures from a 37°C incubator to a 16°C incubator after OD₆₀₀ 0.5–0.6 was reached. Cell pellets were harvested by centrifugation as described above, weighed, frozen in liquid nitrogen and stored at −80°C until used for purification. Some variation in expression levels was observed using different colonies, so glycerol stocks were made from the best-expressing colonies for inoculating large-scale expression cultures and these routinely gave the reported expression yields.

Large Scale Purification of HMunc18c or HLMunc18c

Cell pellets (~15–20 g/L) were thawed on ice for 30 min and then homogenised into a 1:10 ratio (wet cell pellet mass: lysis buffer) in Munc18c lysis buffer (25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, 2 mM β-ME, 1% (v/v) Triton X-100, 0.5 mM EDTA) with 100 µL of Bacterial Protease Inhibitor (BioPioneer, Inc., USA), on ice. The cells were homogenised by multiple passes through a 30 mL disposable syringe and lysed by addition of lysoyme [Astral Scientific, Australia] to a final concentration of 400 µg/mL and incubated at 4°C for 1 h. To reduce viscosity, ~13,000 U of DNase (Roche, Australia) and 1 mM MgCl₂ and 1 mM CaCl₂ was added and the solution was incubated for a further 1 h at 4°C with mixing. The cell lysate was centrifuged to remove the cell debris ([JLA 16.25 rotor, AVANTI centrifuge (Beckman Coulter, USA), 13,500 g, 30 min, 4°C].) and the supernatant was mixed with Ni-chelated PrepEase™ resin (USB Corporation, USA) pre-equilibrated with wash buffer (25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, 2 mM β-ME) and incubated for 2 h at 4°C with gentle mixing. After incubation, the beads were loaded into a gravity column (Maxi Column, GT Bioscience, USA) and washed with 50 mL of the Tris-HCl wash buffer, followed by 50 mL of Tris-HCl wash buffer containing 25 mM imidazole. After washing, the protein was eluted in 1 mL fractions in elution buffer (Tris-HCl buffer with 300 mM imidazole). Eluted protein was analysed on SDS-PAGE (described below) and fractions containing Munc18c were pooled, concentrated to a total volume of 6 mL [10 kDa MWCO centrifugal concentrator (Amicon Merck, Germany) and injected onto a pre-equilibrated Superdex 200 16/60 (8200) size exclusion chromatography (SEC) column on an AKTA FLPC system (GE Healthcare, UK) in SEC Buffer (25 mM HEPES pH 8.0, 200 mM NaCl, 2 mM β-ME, 10% (v/v) glycerol). Fractions containing Munc18c were pooled, concentrated (as before) and injected onto a MonoS cation exchange 5/50 column (pre-equilibrated with buffer A) and purified using a salt gradient from 0%–80% Buffer B (25 mM HEPES pH 8.0, 1000 mM NaCl, 2 mM β-ME, 10% (v/v) glycerol) over 50 Column Volumes at a flow rate of 1 mL per min. Munc18c eluted at between 15–20% of buffer B. Peak fractions were assessed by SDS-PAGE, and highly homogeneous fractions were pooled and concentrated to the desired protein concentration and stored at −80°C.

Large Scale Purification of detagged HTMunc18c

To produce Munc18c lacking a His₆ fusion tag, HTMunc18c was expressed and purified to the point of elution from the PrepEase™ resin, as described above for HMunc18c and HLMunc18c. The eluted HTMunc18c was mixed with TEV protease [47] (0.1 mg protease per 10 mg of protein) was placed in 6–8 kDa MWCO dialysis tubing (Spectrum Lab, Inc. USA) and dialysed overnight against SEC buffer at 4°C incubated in 6–8 kDa MWCO dialysis tubing (Spectrum Lab, Inc. USA). The resulting mixture (HTMunc18c, de-tagged Munc18c, His₆-TEV, His₆) was collected from the tubing and incubated with equilibrated PrepEase™ beads in HEPES wash buffer (25 mM HEPES pH 8.0, 300 mM NaCl, 10% (v/v) glycerol, 20 mM β-ME, 10 mM imidazole) at 4°C for 30 min to separate de-tagged Munc18c (in solution) from His₆-TEV, His₆ and any remaining HTMunc18c (which should all be bound to the resin). The resin was placed in a gravity column, and the flowthrough containing the de-tagged Munc18c was collected. Purity of the de-tagged Munc18c was assessed by SDS-PAGE. Fractions containing the de-tagged protein were pooled and injected onto the pre-equilibrated Superdex-200 16/60 column in SEC buffer on an AKTA FPLC™ system (GE Healthcare, UK). Peak fractions were collected, analysed on SDS-PAGE before pooling and concentrating fractions containing Munc18c and storing at −80°C as described above.

Purification of Syntaxin4 (Sx41-275-His)

C-terminally His₆-tagged Sx4 C141S (residues 1-275) (Sx41-275-His) was produced as described previously [33–35,44].
Interaction of Munc18c with Sx41-275-His and with assembled SNARE complex

To determine whether the Munc18c recombinant protein expressed in E. coli was functional, an in vitro binding assay with the cognate t-SNARE binding partner Sx4 was performed. In this assay, purified Sx41-275-His protein (30 μg) was incubated with 50 μL TALON resin pre-equilibrated with 100 μL of binding buffer (25 mM HEPES pH 8, 300 mM NaCl, 2 mM βME, 10% (v/v) glycerol, 10 mM imidazole pH 7.5, 0.05% (v/v) Triton X-100) for 2 h at 4°C. To remove unbound protein, the beads were washed three times with 500 μL wash buffer (binding buffer with 20 mM imidazole). The immobilised Sx41-275-His was then mixed with 50 μg purified detagged Munc18c in binding buffer and incubated at 4°C with slow mixing for 30 min, 60 min, 120 min and overnight. After incubation, the beads were washed three times with 200 μL of wash buffer. For the negative control, purified de-tagged Munc18c protein (30 μg) alone was incubated with beads in the same manner overnight at 4°C. After washing, beads were mixed with SDS-loading buffer (50 μL) and samples were denatured at 95°C for 5–10 min prior to loading onto a 4–12% Nu-PAGE Bis-Tris SDS-PAGE gel.

The binding of Sx41-275 to recombinant mouse Munc18c expressed in E. coli and recombinant mouse Munc18c expressed in insect cells (HLMunc18cS66) [44] was also determined by monitoring the intrinsic tryptophan fluorescence (excitation 280 nm and emission 310–400 nm) using a Synergy H1-BioTech plate reader. As Sx41-275 does not contain any tryptophan residues, any change in overall fluorescence is likely due to conformational changes in Munc18c upon binding Sx4. All proteins were buffer exchanged into 25 mM HEPES pH 8.0, 300 mM NaCl to remove β-ME using G-25 NAP columns (GE Healthcare, UK). The fluorescence was measured for buffer alone (25 mM HEPES pH 8.0, 300 mM NaCl) and then for each of the purified proteins (HMunc18c, HLMunc18cS66, or Sx41-275-His) at a concentration of 500 nM. The tryptophan fluorescence spectrum was then measured for HMunc18c/Sx41-275-His and HLMunc18cS66/Sx41-275-His (with each protein at 500 nM, in a 100 μL reaction volume). The change in the intrinsic fluorescence upon binding of Munc18c to Sx4 was monitored for each sample in triplicate.

The ability of purified Munc18c to interact with assembled SNARE complex was assessed using the protocol described previously [35]. Briefly, purified SNARE proteins were purified, mixed in a 1:1:1 molar ratio and incubated overnight at 4°C. TALON beads were added to pull down the SNARE complex through interaction with the His6 tag of Sx41-275-His. The beads were washed three times in wash buffer 1 containing 0.1% (v/v) Triton X-100 and then incubated for 2 h at 4°C with de-tagged Munc18c. Beads were washed a further three times in the same wash buffer, mixed with loading dye, boiled for 10 min and bound proteins analysed by reducing SDS-PAGE.

Isothermal Titration Calorimetry (ITC)

ITC experiments were carried out at 298 K using an iTC200 (Microcal) instrument to assess the thermodynamics of Munc18c and Sx4 interaction. The proteins (HMunc18c or Sx41-275-His) were purified as described above and buffer exchanged into ITC buffer (25 mM HEPES pH 8, 200 mM NaCl, 10% (v/v) glycerol and 2 mM β-ME) by gel filtration prior to measurements. Sx41-275-His at a concentration of 200–220 μM was titrated into 20–30 μL of HMunc18c in the cell. Injection volumes of 2.8 μL were used for all titrations. The heat released was measured and integrated using the Microcal Origin 7.0 program using a single site binding model to calculate the equilibrium association constant Ki (1/Kd), enthalpy of binding (ΔH) and the stoichiometry (n). The Gibbs free energy (ΔG) was calculated using the equation ΔG = -RTln(Ki); binding entropy (ΔS) was calculated by ΔG = ΔH – TSΔS. Four replicates were used to generate mean and standard error of the mean (SEM) values.

Purification of the Munc18c/Sx41-275-His complex

Recombinant un-tagged Munc18c and Sx41-275-His proteins were expressed in bacteria as described above. The lysates were mixed in a 3:1 (Munc18c:Sx4) volume ratio to give an estimated molar excess of Munc18c (assuming Munc18c expression ~3 mg/L; Sx41-275-His expression ~3–4 mg/L) and the mixture was incubated on ice for 1–2 h. The mixed lysates were cleared by centrifugation using a JA 25.5 rotor in an AVANTI centrifuge (Beckman Coulter, USA) at 18,500g for 40 min, 4°C. The mixed cleared lysates (200 mL) were then added to TALON affinity beads (1 mL) equilibrated in binding buffer (25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 2 mM β-ME, 0.1% (v/v) Triton X-100), to which 100 μL of Bacterial Protease Inhibitor (BioPioneer, Inc., USA) and ~15,000 U of DNase (Roche, Australia) was added, and this mixture was then incubated at 4°C for 2 h with mixing. The beads were then washed with 150 mL wash buffer 1 (25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 2 mM β-ME, 0.1% (v/v) Triton X-100, 10 mM imidazole) followed by 150 mL wash with wash buffer 2 (wash buffer 1 without Triton X-100). The protein bound to beads was eluted in 1 mL fractions with elution buffer (25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 2 mM β-ME, 300 mM imidazole). The eluted protein was then concentrated using a 10 kDa MWCO concentrator (Amicon, Merck, Germany), injected (5 mL) and purified on a Superdex200 16/60 column on an AKTA FPLC system pre-equilibrated in SEC buffer (25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% (v/v) glycerol) and analysed by SDS-PAGE.

Analytical Size Exclusion Chromatography (SEC)

To assess the homogeneity and stoichiometry of Munc18c/Sx4 complexes an analytical grade Superdex200 10/300 GL column (GE Healthcare, UK) was pre-calibrated with the following molecular mass standards: beta-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa); carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa) (Sigma Aldrich, USA). Proteins alone (HMunc18c, Sx41-275-His) or in complex (un-tagged Munc18c: Sx41-275-His complex) were buffer exchanged into 25 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% (v/v) glycerol and concentrated to 3–5 mg/mL using 3 kDa (for Sx4), 10 kDa (for Munc18c) and 30 kDa for complex) MWCO concentrator (Amicon, Merck, Germany). For analysis, 500 μL each of sample was injected onto the column (pre-equilibrated in the same buffer) and analysed at a flow rate of 0.5 mL/min with column pressure 1.5 MPa. Peak fractions with the correct apparent molecular mass (based on molecular mass standard calibration) were collected, analysed by SDS-PAGE and stored at 4°C for further analysis.

Multi-angle light scattering with SEC (SEC-MALS) was performed at room temperature. A sample (500 μL) of purified HMunc18c at a concentration of 2.5 mg/mL was injected onto an S200 10/300 GL analytical column attached to a mini Dawn laser light scattering photometer and Optilab DSP interferometric refractometer (Wyatt Technology, USA). The column was pre-equilibrated with buffer containing 25 mM Tris-HCl pH 7.5, 300 mM NaCl, 2 mM βME, 10% glycerol. Bovine serum albumin (BSA, Sigma) was used as an isotropic scatterer for detector normalisation. Mass estimation was determined by Debye fitting.
Results
Optimization of recombinant Munc18c production from bacterial culture

The production of recombinant Munc18c from a bacterial expression host has been reported previously (Table 1). We were interested in using bacterially expressed Munc18c for structural and biophysical experiments that require mg quantities of highly purified protein. However, to the best of our knowledge, the yield of protein using these bacterial expression systems was not reported, so it was not clear whether these methods would suffice for our requirements. Following the lead of Brandie et al. (2008) we tested Munc18c expression using the same approach, by co-expressing mouse HMunc18c (native DNA sequence) with GroEL/GroES in M15 E. coli cells at 25°C. The media was used by Brandie et al. (2008) to express Munc18c was not explicitly stated; we used LB media. Under these conditions, we observed that a band corresponding to HMunc18c was barely discernible on a gel, suggesting very low-level expression, albeit with few if any contaminants (Fig. 2B). Expression of the codon-optimized gene was then assessed in LB and TB media with IPTG induction in E. coli strain, BL21, at 25 or 20°C (Fig. 3A–D). Under these conditions, the BL21 strain using LB media was clearly superior to the M15 cells, showing a much higher level of expression with far fewer contaminants (Fig. 3A and 3B). Using the codon-optimized gene in BL21 cells, with ZYP-5052 auto-induction media (Fig. S2A, B), the yield was increased further using ZYP-5052 media (to between ~1–2 mg purified protein from 1 L culture, for different constructs), though with much higher levels of contaminants (Fig. 3G). For the remaining analyses, we chose to use ZYP-5052 auto-induction media in combination with expression at 16°C after cultures reached OD600 0.6.

Next, protein harvesting and purification were optimized. Initially, cell lysis was performed using sonication but this sometimes resulted in lower molecular weight bands on gels after elution from affinity beads (Fig. S2A). To overcome this, a gentler method was developed whereby cells were homogenised in cell lysis buffer at room temperature for 30 min followed by 1 h incubation with lysozyme at 4°C with mixing. This reduced the level of contaminants for HMunc18c (Fig. S2B) and all subsequent purifications were treated in this way.

SDS-PAGE of HMunc18c from SEC peak fractions generally revealed lower molecular weight contaminants (Fig. 4A and 4B). MALDI TOF/TOF mass spectrometric analysis of the tryptic peptides arising from these bands allowed the identification of the contaminants as bacterial SlyD (~21 kDa, a protein rich in histidine), and bacterial 50S ribosomal protein (~30 kDa) (Mascot Peptide Mass Fingerprint as available at www.matrixscience.com). The pl of Munc18c (6.3) was sufficiently different to those of the contaminants (pI 4.9 for SlyD and pI 10.9 for 50S) that it was thought these could be separated by ion exchange chromatography (IEC). Indeed, an IEC step following SEC was able to separate the contaminants (Fig. 4C and D), and yielded ~2 mg purified HMunc18c per L of bacterial cell culture.

Similar optimised expression and purification methods were applied to HTMunc18c and HLHMunc18c (Fig. S3 and S4). However, IEC was not used for HTMunc18c. Instead the His tag was removed by TEV-protease cleavage and the cleaved product separated from the cleared His tags and histidine rich contaminant proteins using a reverse IMAC step. The final yield of purified de-tagged HTMunc18c (TEV protease treated), and of purified HLHMunc18c (~1 mg per L, for 1–2 L cultures) was a little lower than for HMunc18c.

Recombinant HMunc18c from bacterial cultures is monomeric and functional

Purified HMunc18c was analysed on an analytical Superdex 200 column (GL 10/300) to assess its homogeneity in solution. HMunc18c eluted as a major peak (Fig. 5A) at a volume consistent with a 68 kDa protein. SEC-MALS analysis of the purified bacterially expressed HMunc18c gave an estimated mass of 68.8 kDa (±0.3%) confirming that the protein was monomeric (Figure 3B) (theoretical mass for monomer, 68,181 Da). Moreover, purified HLHMunc18c expressed using either bacterial or baculovirus expression systems had equivalent traces on SEC chromatograms (Fig. S5).
To test whether the recombinant HMunc18c expressed in E. coli cultures was functional, its ability to interact with cognate SNARE partner Sx4, was tested. In an in vitro pull down assay, Sx41-275-His was C-terminally immobilized onto affinity beads and incubated with Munc18c (de-tagged HTMunc18c). The results showed that Munc18c expressed in E.coli BL21 cells was pulled down by Sx41-275-His on beads within the first 30 min of incubation (Fig. 6A).

The Munc18c/Sx4 interaction was further evaluated by intrinsic tryptophan fluorescence. Sx4 does not contain any tryptophan residues, whereas Munc18c has six tryptophan residues. Hence, fluorescence changes upon mixing the two would be indicative of interactions and conformational changes in Munc18c. Fluorescence was measured after mixing HMunc18c and Sx41-275-His (500 nM). The spectra of HMunc18c only and Munc18c:Sx41-275-His complex using Munc18c expressed either in E.coli (HMunc18c) or in insect Sf9 cells (HLMunc18cSf9) reveal similar changes (Fig. 6B) indicating that bacterially expressed mouse Munc18c behaves similarly to insect-cell expressed mouse Munc18c.

To further explore the interaction, we measured the thermodynamic parameters for the interaction between Munc18c and Sx41-275 using ITC. The dissociation constant for the interaction ($K_d$) was 104 ± 43 nM (Table 2) (Fig. S6), which compares closely to the previously reported affinity using insect cell expressed Munc18c [33]. The Munc18c:Sx41-275-His complex was formed by mixing the lysates of E. coli expressed mouse Munc18c (un-tagged) and Sx41-275-His. The mixed lysates were clarified and then incubated with TALON beads, to pull down Sx41-275-His and bound protein. Eluted fractions from TALON beads were pooled and analysed by SEC. A major peak eluted at a volume consistent with a 100 kDa protein (the mass of the Munc18c: Sx41-275-His complex) (Fig. 6C). Peak fractions analysed by SDS-PAGE revealed two bands with relative intensities suggesting the formation of a 1:1 stoichiometric heterodimer complex between Munc18c and Sx41-275-His (Fig. 6C). This result indicates that both proteins Munc18c and Sx41-275-His (expressed in bacteria) are correctly folded and functionally competent to form a stable complex.

Discussion

The interaction between Munc18c and Sx proteins is a major focus for understanding the molecular basis of vesicle fusion. Research relating to the molecular mechanism and regulation of this key complex requires the ability to produce milligram quantities of the purified, soluble and folded target proteins using rapid, reproducible and cost-effective methods. Structural studies of Munc18c have used recombinant protein expressed in baculovirus-infected insect cells [33,34,38,44] whereas several biochemical studies have used recombinant Munc18c expressed...
from E. coli cultures [29,39–42,49]. Here, we attempted to optimize the expression of Munc18c in E. coli to enable sufficient yields for structural and biophysical studies.

We succeeded in this goal by making use of the previously reported co-expression of Munc18c with GroEL [29] combined with the following modifications: use of codon-optimized gene, BL21 E. coli cells, auto-induction media and expression at low temperature. Also, by replacing the sonication step with a gentler lysozyme treatment, we were able to minimize the level of lower molecular weight contaminants of Munc18c at the first stage of purification. The identification of the bacterial contaminants enabled us to add an IEC step to the purification and remove the contaminants by taking advantage of their pl values relative to Munc18c. We were able to generate mg quantities of purified Munc18c sufficient for structural and biophysical studies.

Most importantly, the purified Munc18c was monomeric, mono-disperse and functional. We were able to show that Munc18c binds Sx4 robustly using several different approaches: pull downs; intrinsic fluorescence; and ITC. Moreover, the Munc18c: Sx4 heterodimer was co-purified with an
Figure 4. Purification of recombinant HMunc18c expressed in E. coli cultures. A. SDS-PAGE analysis of HMunc18c purification steps. Elution fraction from IMAC, labelled, was injected onto SEC and eluted as shown in Lanes 1–6 (which correspond to the labelled fractions in panel B). B. Elution profile of HMunc18c from SEC. Peak fractions were pooled and injected onto a MonoS column. C. SDS-PAGE of fractions from the MonoS purification step, showing separation of the protein from lower molecular weight contaminants. D. Elution profile of HMunc18c from MonoS.

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Figure 5. Purified HMunc18c is monomeric in solution. A. Elution profile of purified HMunc18c on a calibrated analytical size exclusion chromatography column (S200 10/300 GL). HMunc18c eluted at a volume consistent with a ∼70 kDa protein. Peak fractions were analysed on 4–12% gradient SDS-PAGE (inset). B. Elution profile of HMunc18c examined by SEC-MALS. The horizontal blue line corresponds to the SEC-MALS calculated mass (right axis) plotted with the refractive index indicating the peak (left axis) of the protein in the sample (68,200 Da ± 0.5%).

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apparent 1:1 stoichiometry. The binding affinity for Sx41-275-His was 104 ± 43 nM for Munc18c expressed from bacteria as compared with 95 ± 15 nM for Munc18c expressed in insect cells [33]. These results indicate that Munc18c expressed in bacteria is correctly folded, and functional in its ability to interact with Sx4.

These findings are encouraging for the use of bacterially expressed Munc18c for future protein-protein interaction and structural studies. The cost of expressing the protein in this way reduces the cost of media and consumables by more than a factor of ten. Also bacterial expression and purification can be completed

| Munc18c/Sx4 thermodynamic parameters |
|--------------------------------------|
| In cell | Titrant | ΔH (kcal/mol) | TΔS (kcal/mol) | ΔG (kcal/mol) | Kd (nM) | N     | Reference |
|---------|---------|---------------|----------------|---------------|--------|-------|-----------|
| HMunc18c | Sx41_275-His (32 kDa) | −11.0 ± 0.4 | 5.6 ± 1.4 | −9.2 ± 0.5 | 104 ± 43 | 0.97  | This work |
| HLMunc18cSf9 | Sx41_275-His | −7.70 ± 0.1 | −1.9 ± 0.1 | −9.5 ± 0.1 | 95 ± 15 | 0.98  | [33]      |

This work
in 2–3 days rather than 15–17 days for insect cell expressed protein. The typical final yield of purified Munc18c (HMunc18c, HTMunc18c, HLHunc18c or untagged Munc18c) expressed in bacterial cultures was between 1–2 mg per L of cell culture as compared to 3–4 mg per L insect cell culture.

**Supporting Information**

**Figure S1** DNA sequences. **A.** codon optimized gene for mouse full-length Munc18c expression in E. coli. **B.** Linker sequence. (TIF)

**Figure S2** Effect of cell-lysis method on HMunc18c purification. SDS-PAGE gel showing eluted HMunc18c from IMAC beads after cell lysis by A. sonication or B. lysozyme treatment. (TIF)

**Figure S3** Purification of HTMunc18c. **A.** SDS-PAGE analysis of HTMunc18c purification steps. **B.** TEV cleaved (de-tagged) HTMunc18c obtained by reverse IMAC. **C.** Elution profile of the de-tagged Munc18c from SEC (lanes 1–4). (TIF)

**Figure S4** Purification of HLHunc18c. **A.** SDS-PAGE analysis of HLHunc18c IEC fractions. **B.** Elution profile of HLHunc18c from IEC MonoS column. (TIF)

**Figure S5** Comparison of Munc18c produced from insect or bacterial culture. **A.** Overlaid SEC chromatograms for HLHunc18c expressed from Sf9 insect cells (dark blue) or E. coli (light blue). **B.** Samples injected onto the SEC column in panel A, were assessed by Coomassie-blue stained SDS-PAGE. (TIF)

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**Figure S6** ITC raw data. The upper panel shows the raw data from a representative experiment for the ITC measured interaction between HMunc18c (in the cell) and Sx41-275-His (in the syringe). The lower panel shows the integrated and normalised data. (TIF)

**Figure S7** Recombinant Munc18c generated from E. coli expression culture binds to assembled SNARE complex. Coomassie Blue stained SDS-PAGE gel showing the binding of Munc18c (de-tagged) to pre-formed SNARE ternary complex. The input proteins for this experiment are shown on extreme left. The SNARE complex was formed by mixing solutions of purified Sx41-275-His, SNAP23 and VAMP2 and incubating overnight at 4°C. The SNARE complex was then isolated on TALON Co2+ beads. The beads were then incubated for 2 h with de-tagged Munc18c and washed prior to analysis by SDS-PAGE. A sample of the SNARE complex assembled and captured on beads, prior to addition of Munc18c is shown for comparison. (TIF)

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**Author Contributions**

Conceived and designed the experiments: AR RJJ AEW GJK SHH JLM. Performed the experiments: AR RJJ AEW GJK SHH. Analyzed the data: AR RJJ AEW GJK SHH MPC BMC JLM. Contributed reagents/materials/analysis tools: AR RJJ AEW GJK SHH MPC BMC JLM. Wrote the paper: AR RJJ AEW GJK SHH JLM. Contributed critical comments on the draft and the revised version of the manuscript: AR RJJ AEW GJK SHH MPC BMC JLM.
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