Ability of human SNAP-23 to generate high molecular weight SDS-resistant ternary SNARE complexes is influenced by C-terminal coil content

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ABSTRACT

Using *in vitro* protein complex formation assay, ability of SNAP-25 isoforms to generate SDS-resistant ternary SNARE complexes with Syntaxin-1 and VAMP-2 was investigated. Major SNAP-25 family proteins were found to generate heat-resistant ternary complexes with varying efficiency. Compared to human SNAP-25, its non-neuronal counterparts SNAP-23 and SNAP-29 formed lower amounts of ternary complexes. Changing Pro182 in human SNAP-23 to Arg182 (SNAP-23 P182R) improved its ability to bind partners and form complexes. *In silico* analysis of C-terminal helical content in various SNAP-25 family members showed that except human SNAP-23, all others displayed secondary α-helical conformation. We also report that human SNAP-29 is resistant to the proteolytic action of botulinum neurotoxin A even when applied at large concentration.

1. Introduction

Members of the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) superfamily are abundantly and widely expressed proteins involved in intracellular membrane fusion events [1]. The SNARE proteins can be divided into two large sub-groups depending on their location; those residing on vesicle membranes are called the v-SNAREs (vesicle-SNAREs) and ones present on the target membrane are called t-SNAREs (target-SNAREs). Neuronal v-SNARE VAMP-2 (also called Synaptobrevin) and t-SNAREs Syntaxin-1 and SNAP-25 were discovered first as important protein components in regulated synaptic exocytosis [2,3]. In mammalian cells, SNAP-25 and its homolog SNAP-23 represent the major t-SNAREs. SNAP-25 is expressed predominantly in neurons and neuroendocrine cells, while SNAP-23 has a ubiquitous expression. SNAP-23 is the non-neuronal homolog participating in membrane fusion in both neuronal and non-neuronal tissues [4]. Both SNAP-25 and SNAP-23 interact with members of t- and v-SNARE families forming ternary SNARE complexes that are critical intermediates during membrane fusion [5,6]. Using recombinant enzymatic domain (light chain) of tetanus and botulinum neurotoxins, the central role of SNARE proteins in membrane fusion events was established [7,8]. While neuronal SNAREs are extremely sensitive to cleavage by clostridial neurotoxins, many non-neuronal SNAREs are found toxin-resistant [9,10].

The heterotrimeric SNARE complexes have been a subject matter for many studies. Structure of the heterotrimeric SNARE complex formed between Syntaxin 1, VAMP-2 and SNAP-25 is a parallel four-helical bundle [11]. In this ternary complex, one helix each is provided by Syntaxin and VAMP while two helices, one located at N-terminus and other at C-terminus, are provided by SNAP-25. The complex is a coiled-coiled structure that imparts high thermal and chemical stability exemplified by its remarkable stability in SDS-containing buffers even at 80 °C [12]. Parallel four-helical bundle structure is thought to be a common feature in a stable and functional trimeric SNARE complex mediating membrane fusion.

Despite high degree of conservation in their SNARE interacting C-terminal region, SNAP-25 and SNAP-23 show differences in binding to SNARE partners. It is observed that thermal stability of SNARE complexes containing SNAP-23 is generally lower than those formed with SNAP-25 [13]. In a study conducted by Montana et al. [14], using force spectroscopy to assess protein complex stability, it was reported that complexes containing SNAP-23 displayed lower stability than those with SNAP-25, and the stability difference may explain their differential ability to support exocytotic burst. A relationship between partner SNARE binding, ternary complex stability and efficiency in exocytosis was observed by several workers. In insulin-secreting pancreatic cell line HIT-T15, human SNAP23 was able to replace SNAP-25 partially [15] and this was attributed to better SNARE binding property of SNAP-25. In rat mast cells, a small expression of human SNAP-23 significantly increased the rate of exocytosis [16] due to better SNARE binding ability

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of human SNAP-23 than rat SNAP-23. Structural features in SNAP-25 and SNAP-23 proteins that influence their binding to partner SNARES and subsequently determine SNARE complex stability is not well understood.

During our investigation on interaction of human SNAP-23 with Syntaxin-4 and VAMP-2, we observed that its ability to form SDS-resistant ternary SNARE complexes was lower than human SNAP-25. Further examination showed that Proline[182] in human SNAP-23 contributed for its diminished interaction with partner SNAREs and interaction with botulinum neurotoxin toxin light chain A. *In silico* analysis showed that human SNAP-23 is devoid of coiled α-helical content in the C-terminus, attributable to Pro[182] residue in the region. Thus, poor interaction of human SNAP-23 with partner SNAREs and botulinum neurotoxins can be explained due to lack of coiled structures in its C-terminal end.

2. Material and methods

2.1. Plasmid constructs

Clones of full-length human SNAP-25, SNAP-23, SNAP-29 and mouse SNAP-23 were made in pSP72 vector (Promega, Heidelberg, Germany). Point mutants of human SNAP-25 (R172P) and human SNAP-23 (P182R) were created by PCR and mutation was confirmed by DNA sequencing [9]. Full-length botulinum light chain toxins A and E, and rat VAMP-2 with C-terminal 6x Histidine tag were cloned in pET-9c vector. Full-length Glutathione-S-transferase (GST) and GST-human Syntaxin-1a were cloned in pGEX-2T vector (Pharmacia, Freiburg, Germany).

2.2. Expression and purification of recombinant proteins

Recombinant proteins were produced in *E. coli* by standard protein expression protocols [8,9] and expressed proteins were purified using affinity chromatography resins. Purified proteins were dialyzed against assay buffer (150 mM K glutamate, 10 mM HEPES-KOH, pH 7.2), frozen in liquid nitrogen, and stored at −70 °C.

Purified botulinum neurotoxin light chain proteins were characterized as described earlier [8].

2.3. In vitro transcription and translation

[^{35}S] methionine radiolabeled human SNAP-25, SNAP-23, SNAP-29 or mouse SNAP-23 proteins were synthesized *in vitro* from pSP72 transcription plasmid using TNT® Quick Coupled Transcription/Translation System (Promega) following manufacturer's instructions. T7 Polymerase was included to initiate transcription reaction. Translations were performed using 0.1 μg circular plasmid DNA, T7 Polymerase, amino acid mixture lacking methionine and [^{35}S] methionine (24 μCi, 1200 Ci/mmol; ICN Biomedicals, Irvine, CA, U.S.A.) in a total volume of 25 μl. Radiolabeled proteins were prepared fresh ahead of each experiment.

2.4. In vitro SNARE complex formation assay

In vitro SNARE complex assay was done as described by Hayashi et al. [12] in a total volume of 100 μl. The assay contained 0.1 nmol of GST-Syntaxin 1 and 0.8 nmol of His6-VAMP-2 and 1 μl of *in vitro* translated [^{35}S] methionine-labeled human SNAP-25, human SNAP-23, mouse SNAP-23, human SNAP-29 or their mutants. The assay buffer used was 150 m*M* K glutamate, 10 m*M* HEPES-KOH, pH 7.2. The contents were mixed for 4 h at 4 °C and 20 μl of washed Glutathione Sepharose beads (50% w/v) was added. The contents were mixed further for 1 h and the beads were collected by brief centrifugation. After extensive washing of the beads with assay buffer, 20 μl of SDS-PAGE sample buffer (120 mM Tris-HCl, pH 6.75, 10% β-mercaptoethanol, 4% sodium dodecyl sulfate, 20% glycerol and 0.014% bromophenol blue) was added to each tube. Tubes were kept either at 37 °C for 15 min

or heated to 96 °C for 5 min to release the bound proteins. Samples were further analyzed in 12% SDS-PAGE gels, followed by auto-fluorography to visualize bands. Control incubations were carried out simultaneously using *in vitro* translated human SNAP-25 wild type protein mixed with Glutathione Sepharose beads. At the end of incubation, an aliquot of the supernatant fraction was analyzed by SDS-PAGE.

2.5. Toxin treatment

Toxin cleavage of recombinant SNAP-25 isoform proteins was done as described earlier [9]. The cleavage assay contained 1 μl of the translation mixture of [^{35}S] methionine-labeled human SNAP-25 or its mutant or human SNAP-23 along with the respective light chain recombinant protein (BoNT/A or E). The reaction was incubated for 60 min at 37 °C in a total volume of 10 μl of toxin assay buffer. At the end of incubation, 1 μl of the reaction mixture was mixed with SDS-PAGE sample buffer. Samples were boiled for 3 min and subjected to SDS–PAGE electrophoresis using 15% gels. Proteins were visualized after staining with Coomassie Blue using a LAS-3000 imaging system and radiolabeled samples by fluorography using a BAS-1500 phosphoimager (Fuji Photo Film, Japan) and evaluated by the AIDA 2.11 program.

3. Results

Ternary SNARE complexes are believed to be central in the process of membrane fusion and it is well known that SNAP-25 forms complexes with multiple Syntaxins and VAMPs. To evaluate efficiency of SNAP-25 orthologs to associate with partner v-SNAREs and form heat-stable ternary SNARE complexes, we performed *in vitro* ternary complex formation assays with human Syntaxin 1 and VAMP-2 as partner SNAREs. In this assay, ^{35}S-methionine labeled SNAP-25 proteins generated by *in vitro* translation were incubated with recombinant His$_6$-VAMP-2 and GST- Syntaxin-1 to allow complex formation. Ternary complexes were captured by adding Glutathione Sepharose beads into the assay mixture. After extensive washing of the capture beads with buffer, bound complexes were released by incubation in buffers containing SDS at 37 °C for 15 min or 96 °C for 5 min. Released proteins were analyzed by SDS-PAGE followed by phosphoimager analysis. Results of this experiment are presented in Fig. 1. Formation of SDS-stable protein complexes was seen with all members of SNAP-25 protein family we tested (Lanes 2, 4, 6 & 8). However, differences were seen especially between SNAP-25 and SNAP-23 in terms of quantity and types of complexes formed. In SDS-PAGE gels, it was clearly seen that SNAP-25 generated complexes ranging from 66 to 220 kDa in molecular weight while human SNAP-23 generated complexes confined to a lower range of 66–80 kDa. We next examined formation of high molecular weight SNARE complexes with mouse SNAP-23 and human SNAP-29 in *in vitro* binding assays using Syntaxin-1 and VAMP-2 as partners. Mouse SNAP-23 generated heat-resistant SNARE complexes in a molecular weight range comparable to that of SNAP-25 (100–220 kda). Human SNAP-29 formed lower amount of ternary SNARE complexes, but they had molecular weight of 100 kDa and above. In both mouse SNAP-23 and human SNAP-29, the ternary complexes were completely dissociated when subjected to elevated temperature. (Lanes 3, 5, 7 & 9).

Earlier we had reported that human SNAP-23 P182Rmutant was cleaved by light chain of botulinum neurotoxin A and E *albeit* low level [9] and therefore we examined properties of ternary SNARE complexes formed by this mutant. In SDS-PAGE gels, the mutant SNAP-23 showed formation of higher molecular weight complexes in the range of 100–220 kDa and this pattern resembled human SNAP-25. Interestingly, when we used human SNAP-25 R176P mutant protein in SNARE complex assay, formation of high molecular weight ternary complexes of >100 kDa was found diminished. The molecular weight range of complexes became similar to those generated by native human SNAP-23.

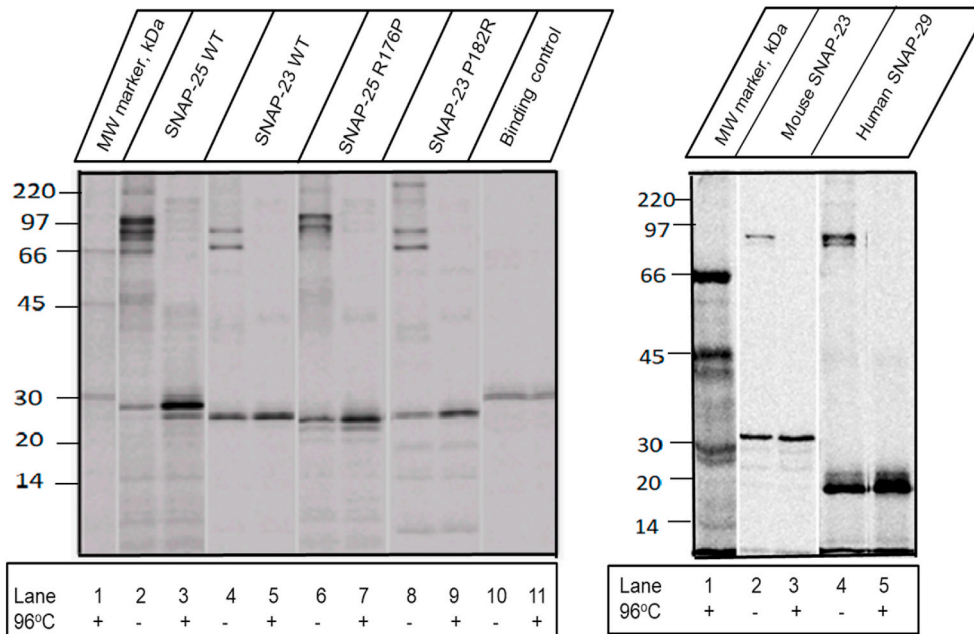To confirm differences observed between human SNAP-25 and

Fig. 1. Ability of SNAP-25 protein family members to form high molecular weight SDS-resistant ternary complexes. *In vitro* SDS-resistant ternary complex formation assay was carried out as described in methods. Proteins were separated using 15% polyacrylamide SDS-containing gels. For complex formation assays, incubation contained radio-labeled SNAP-25 proteins generated in *in vitro* translation reactions using rabbit reticulocyte system, recombinant human GST-Syntaxin-1 and rat His$_6$-VAMP-2. (A) Lane: 1: ^{14}C-labeled protein markers in kDa; Lanes 2 & 3: complexes formed with human SNAP-25 wild type; Lanes 4 & 5: complexes formed with human SNAP-23 wild type; Lanes 6 & 7: complexes formed with human SNAP-25 R176P mutant Lanes 8 & 9: complexes formed with human SNAP-23 P182R mutant; Lanes 10 & 11: control binding experiment using radio-labeled human SNAP-25 wild type protein and Glutathione Sepharose beads followed by analysis of the supernatant fraction by SDS-PAGE human GST Syntaxin1a. **(B)** Lane 1: ^{14}C-labeled protein marker Lane 2&3: ternary complexes formed with human SNAP-29; Lanes 4&5: ternary complexes formed with mouse SNAP-23.

SNAP-23, we did quantitative analysis of the radioactivity incorporated in high molecular weight ternary complexes by phosphoimager analysis of SDS-PAGE gels. We first compared complex formation between native SNAP-25 and SNAP-23 proteins. Lower amount of radioactivity incorporation (~50% less) was seen in complexes containing native human SNAP-23 protein compared to human SNAP-25 (Fig. 2: Lanes 1 & 5). In

the next set of experiments, we compared radioactivity incorporation in ternary complexes generated in presence of specific mutant proteins, namely human SNAP-25 R176P and human SNAP-23 P182R. This experiment was based on our earlier observation that human SNAP-23 P182R was cleaved by high concentration of Botulinum toxin types A and E, likely due to improved protein secondary structure at the C-terminal region [9]. Indeed, ternary complexes containing human SNAP-23 P182R mutant contained higher radioactivity than the wild type SNAP-23 protein with label incorporation reaching levels almost equal to that of native human SNAP-25 (Fig. 2, compare lanes 1 & 7). When we measured radioactivity in complexes formed with human SNAP-25 R176P mutant, it was found 25% lower than incorporation achieved with wild type SNAP-25 protein. The modest impact after introducing proline in SNAP-25 highlights role for residues other than Arginine176 in SNAP-25 in efficient SNARE complex formation. Work done by Fang et al. [17] has shown that several positively charged amino acids in the C-terminus of SNAP-25 participate in tight SNARE complex association. In summary, our radioactive binding data supports gel electrophoresis data, and shows differences in pattern and quantity of SDS-resistant ternary SNARE complexes formed by human SNAP-23 and SNAP-25 proteins.

Therapeutic applications of light chains A and E targeting SNAP-23 in disease states associated with secretory dysfunctions is an emerging area of research and our laboratory is engaged in generating light chain mutant toxins active on SNAP-25 and SNAP-23 orthologs [18,19]. As part of this investigation, we first studied the toxin-target interaction using the human SNAP-25 R176P mutant in toxin cleavage assays (Fig. 3A&B). We found that human SNAP-25 R176P was highly resistant to toxin action. In standard toxin cleavage assay, while the wild type SNAP-25 was completely cleaved after 60 min of incubation with A and E light chains, proteolysis of mutant protein was poor (~60% and ~20% respectively, for BoNT/A and E). We also found that for complete cleavage of the mutant SNAP-25 protein, considerably higher light chain concentration was required in the assay. Separately in protein binding assays, we found that light chains of A and E do not bind efficiently to the SNAP-25 R176P protein (data not shown). Since human SNAP-29 generated high molecular weight SNARE complexes like SNAP-25, we tested its sensitivity to Botulinum toxin light chain A action (Fig. 3C).
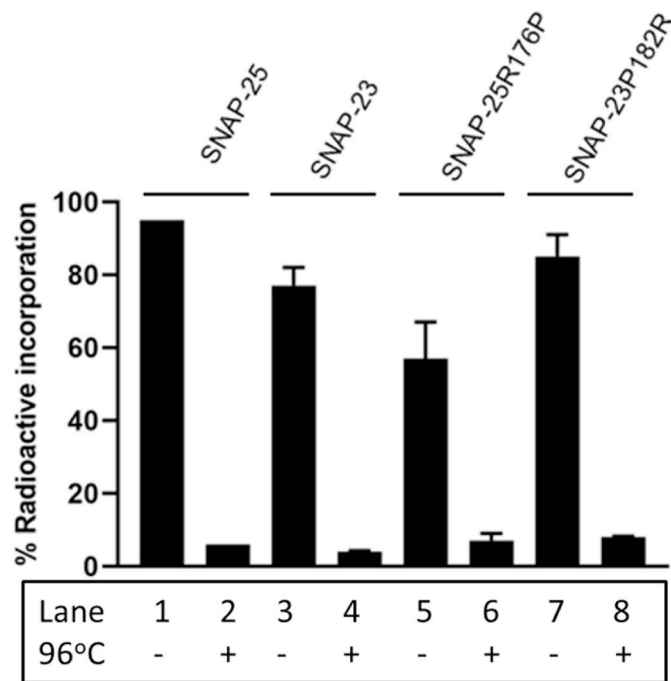

Fig. 2. Quantitative analysis of the radioactivity incorporated in the high molecular weight SNARE ternary complexes formed by human SNAP-25, human SNAP-23 and their mutants with human Syntaxin-4 and rat VAMP-2. *In vitro* complex formation assay using radio-labeled proteins and quantitation of ternary SNARE complexes by SDS-PAGE was carried out as described in methods. Values shown are mean ± SD from three independent experiments.

Fig. 3. Cleavage kinetics of SNAP-25 R176P mutant with botulinum neurotoxin light chains A and E, and resistance of human SNAP-29 to toxin action. Radiolabeled human SNAP-25 and its mutant generated by *in vitro* translation reaction using rabbit reticulocyte lysate was used as substrate and incubated with 1 nM of recombinant toxin light chains for varying periods of time. Cleavage of substrate was determined by SDS-PAGE and quantified by phosphoimager analysis. Results are mean values of duplicate estimations and representative of two independent experiments. For evaluating toxin action on mouse SNAP-23 and human SNAP-29, radiolabeled substrates were incubated with purified toxin light chains (1 μM final concentration) for 1 h at 37 °C before analysis. Arrow in Panel C indicates truncated protein generated by toxin action.

Even after prolonged incubation at high toxin concentration, human SNAP-29 remained intact while mouse SNAP-23 showed partial cleavage (<1% of total substrate cleaved).

The C-terminal SNARE motif in SNAP-25 is suggested to function as binding site for partner proteins and botulinum neurotoxin light chains [20] and it was conceivable that different SNAP-25 protein isoforms have varying amounts of secondary structure in their C-terminal regions.

To pursue this hypothesis, we used a simple approach of using COILS program [21] for predicting coiled-coil secondary structure in various SNAP-25 isoforms. COILS is a *in silico* program that compares a sequence to a database of known parallel two-stranded coiled-coils to derive a similarity score. By comparing this score to the distribution of scores in globular and coiled-coil proteins, the program calculates the probability that the sequence will adopt a coiled-coil conformation. The results of *in*

Fig. 4. Sequence comparison and analysis of coil content in SNAP-25 family proteins. (A) Sequence comparison was performed using Clustel Omega. (B) Coil content was analyzed using COIL program.

silico analysis are presented in Fig. 4. Except human SNAP-23, other SNAP-25 homologs exhibited high propensity to form coiled structures in the C-terminal region. Human SNAP-23 was characterized by almost a complete absence of secondary structure which was reinstated in human SNAP-23 P182R (data not shown). As a corollary, human SNAP-25 R176P mutant exhibited significant loss of coiled structure in its C-terminus (data not shown). It is thus evident from *in vitro* and *in silico* data that secondary structural features in the C-terminal region of human SNAP-23 influences the protein's ability to efficiently bind SNARE partners and generate high molecular weight complexes. Additionally, this region also has great influence on binding of botulinum neurotoxin light chains as evident from our studies with SNAP-23 and SNAP-25 mutants.

4. Discussion

A major objective of this work was to understand molecular differences between human SNAP-25 and human SNAP-23 in their ability to bind SNARE partner proteins forming ternary complexes. While several reports exist on ability of SNAP-25 to form stable ternary SNARE complexes, such information on SNAP-23 analogs is scanty. Ability of SNAP-23 proteins to form promiscuous binary complexes is well known [13]; however ternary SNARE complexes containing SNAP-23 is not studied in detail despite data showing functional inequality between SNAP-25 and SNAP-23 [15,16]. Convincing data exists to show differences in the stability of SNAP-25 and SNAP-23 containing ternary complexes and link it to functional differences between the two proteins in exocytosis. It is also evident that SNARE complexes of varying stability are generated in different situations such as rapid and slow secretions, and contributes in maintaining homeostasis during secretory events.

Structural and functional relevance of SDS-resistant ternary SNARE complexes in living cells is not yet clearly understood. It is proposed that they simply represent different stages of protein assembly during ternary SNARE complex formation. In PC12 cell membranes, using Western blot techniques, Kubista et al. [22] reported presence of several SDS-resistant ternary SNARE complexes distributed over a molecular weight range of 60–250 kDa. The behavior of higher molecular weight ternary SNARE complexes in this cell type was interesting. Upon depolarization of cells with K+ ions, disappearance of complexes with higher molecular weight of ~ 230 kDa was evident which reappeared upon repolarization. During this time levels of another prominent SNARE complex of molecular weight of ~100 kDa either increased or remained unchanged. In our study, we observed clear differences in molecular weight distribution of ternary SNARE complexes mediated by human SNAP-25 and SNAP-23. SNAP-25 was able to generate complexes of higher molecular weight over a wider range than other isoforms and this might indicate its ability to form complex SNARE assemblies functionally designed for rapid exocytotic events such as neurotransmitter release. Our results support the view that living cells contains SDS-resistant SNARE complexes of different structures and functions to participate in different stages of exocytosis.

Involvement of heptad repeat motifs called the SNARE motif in productive target-botulinum toxin interaction is well known. Study done by Washborne et al. [20], showed that efficient proteolysis of SNAP-25 by BoNT/A and BoNT/E require the most C-terminal motif, called S4. Between human SNAP-25 and SNAP-23, S4 motif is well-conserved; however, in human SNAP-23 S4 motif most likely lacks secondary structure, thereby affecting toxin-target interaction. Elegant structural studies on SNAP-25 - light chain toxin A complex done by Feltrup et al. [23], have shown that functional flexibility of the C-terminus of toxin light chain is critical for its biological activity. According to the fly-casting model proposed by them, during toxin-target interaction, SNARE motif of SNAP-25 first binds to an exosite (site remote from active site of the toxin protease) on BoNT/A light chain, and then makes multiple contacts with the toxin before approaching the active site. The highly flexible conformation of C-terminal region of light chain toxin A

allows rapid initial binding of SNAP-25 to the exosite, which leads to further increase in flexibility of the light chain resulting in catalytic activity. Toxin resistant SNARE proteins may lack ability to induce such a functional flexibility in the toxin light chain due to poor initial binding.

Among the mammalian SNAP-25 family members, human SNAP-23 shows the highest homology to SNAP-25 (59% identical and 72% similar at the amino acid level, considering conservative amino acid substitutions). In the C-terminal region consisting of amino acids 160–216, they are 60% identical and 20% similar. Human SNAP-29 has a lower homology to SNAP-25 and SNAP-23 (17% identical and 31% similar to SNAP-23 and 17% identical and 33% similar to SNAP-25). Interestingly, at the C-terminal region, homology is very low between SNAP-29 and SNAP-25 or 23. Despite such high degree of conservation, SNAP-25 and SNAP-23 proteins show differences in binding to SNAREs and regulatory proteins, where their C-terminal region is intimately involved. It is likely that subtle differences in amino acid sequence in the C-terminus, such as Proline182 in human SNAP-23 influences protein interactions.

It is interesting that amongst SNAP-25 homologs, human SNAP-23 had the lowest content in the C-terminal region, and this may have consequences for the structure of ternary SNARE complexes containing human SNAP-23. The proposed structure for neuronal ternary SNARE complex is a four helical bundle, with SNAP-25 contributing two helical coiled-coil motifs (from its N- and C-termini) and Syntaxin-1 and VAMP-2 contributing one coiled-coil each. It is generally believed that non-neuronal ternary SNARE complexes also have similar organization. *In vitro* complex formation studies with purified coiled coil domains of SNAP-23, Syntaxin-4 and VAMP-3 has shown that although hetero tetrameric structures resembling neuronal SNARE complexes are formed, analysis by mass spectrometry showed that they contained only N-terminal coiled-coil domain of SNAP-23 [24]. Low solubility of purified C-terminal coiled-coil domain of SNAP-23 was suggested as a reason for not obtaining crystals containing both N-and C-terminal coiled domains. It is likely that in their experiments, the C-terminal coiled domain of SNAP-23 did not associate at all with other coiled coil components. It is also quite possible that the tetrameric complexes generated in their study contained only N-terminal region of SNAP-23 as complexes were detected by protein staining in SDS-PAGE gels which makes it difficult to distinguish between N-and C-terminal coiled coil domains. Secondary structure data we have presented strongly indicates that drastically reduced α-helical content in the C-terminal region of human SNAP-23 interferes with its ability to efficiently associate with partner SNAREs. In this background, it is tempting to speculate that ternary SNARE complexes containing human SNAP-23 is a triple-helical bundle, with C-terminal coiled coil region of human SNAP-23 largely excluded in the complex. Such complexes may have reduced stability, generate lower molecular weight ternary complexes and contribute to functional differences observed between SNAP-25 and SNAP-23 in human cells.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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