nSec-1 (munc-18) interacts with both primed and unprimed syntaxin 1A and associates in a dimeric complex on adrenal chromaffin granules

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The target-SNARE syntaxin 1A is an essential component of the core machinery required for regulated exocytosis (where SNARE is the soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor). Syntaxin 1A interacts with a variety of other proteins, two of which, *N*-ethylmaleimide-sensitive fusion protein (NSF) and α -soluble NSF attachment protein (α -SNAP) have been suggested to impart a conformational rearrangement on this protein during a reaction referred to as priming. We have studied the effect of the primed state on the binding properties of syntaxin 1A and we have confirmed that primed syntaxin 1A no longer associated with α -SNAP or its cognate vesicle-SNARE, vesicle-associated membrane protein (VAMP). Under such conditions, however, it retained the ability to bind to nSec-1. It has been demonstrated that nSec-1, a regulatory protein also involved in neuronal exocytosis, binds syntaxin 1A with high affinity *in*

vitro, although evidence for this physical interaction occurring *in vivo* has proven elusive. We analysed the subcellular distribution of these two proteins in fractions from bovine adrenal medulla and detected syntaxin 1A and nSec-1 in both plasma membrane and chromaffin-granule fractions. Using a cross-linking approach with chromaffin-granule membranes we detected a putative dimeric complex composed of approx. 54 % total granule membrane nSec-1 and approx. 30 % total syntaxin 1A. The results of this study therefore suggest the possibility of nSec-1 interactions with primed syntaxin 1A and demonstrate a potentially significant interaction of syntaxin 1A and nSec-1 on the membranes of chromaffin granules.

Key words: exocytosis, NSF, α -SNAP, SNARE.

INTRODUCTION

Intracellular fusion of transport vesicles with their target membrane compartments has been shown to involve the concerted action of a vast array of protein factors [1,2]. In particular, a set of proteins termed SNAREs have been focused upon as essential to various intracellular trafficking steps in numerous organisms (where SNARE is the soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) [3]. In pre-synaptic nerve terminals, three SNARE proteins were found to be involved in the regulated secretion of neurotransmitter charged synaptic vesicles with the pre-synaptic plasma membrane and they have subsequently been found to participate in a wide range of regulated exocytotic events [4]. The largely plasma membrane localized target- or t-SNAREs, syntaxin 1A and the 25 kDa synaptosome-associated protein (SNAP-25), form a highly stable SDS resistant trimeric complex with a partner vesicle- or v-SNARE vesicle-associated membrane protein (VAMP), which is predominantly localized to secretory vesicles [5]. The structural organization of the trimeric complex has been characterized [6,7]. It is, however, the cycling of this trimeric SNARE complex which has caused intense debate as to the exact role of the SNAREs in membrane fusion events.

The assembly/disassembly of the neuronal SNAREs is mediated by the soluble chaperone protein *N*-ethylmaleimide-sensitive fusion protein (NSF) and its adaptor protein α -soluble NSF attachment protein (α -SNAP) [8]. Originally, SNARE complex disassembly via NSF/ α -SNAP was believed to represent a late step, following docking of vesicles with the plasma membrane, close to actual bilayer fusion. A wealth of experimental data has since accumulated questioning this view.

One such line of evidence relates to a previously unknown effect of NSF action on the t-SNARE syntaxin 1A. Disassembly of the SNARE complex leads to a so called 'primed' state of the syntaxin molecule [9] which appears to be physiologically significant in that the intrinsic ATPase activity of NSF is stimulated as this transition from unprimed to primed occurs [10,11]. Priming can occur on the membrane of vesicles prior to attachment to target membranes and therefore represents a step that is spatially and temporally distinct from the actual fusion event. In the context of neuronal regulated secretion, primed syntaxin 1A can no longer associate with its cognate v-SNARE, VAMP, or with the NSF adaptor protein α -SNAP [9]. The importance of the priming reaction in membrane trafficking events can be gauged by an appreciation of its evolutionary conservation. In a yeast homotypic vacuole fusion system, the NSF/ α -SNAP homologues have been proven to impart an essential priming function upon vacuoles harbouring only t-SNAREs and this is prerequisite for subsequent fusion of these compartments [12].

In this study, we have attempted to further define the characteristics of the primed syntaxin 1A molecule with an aim to providing a better understanding of the physiological significance of this reaction in the scheme of vesicular membrane trafficking. The data presented provides further evidence for a significant conformational change in syntaxin 1A occurring during priming. Furthermore, we were able to demonstrate specific binding of nSec-1 to syntaxin 1A under both primed and unprimed states of the syntaxin protein. While binding of nSec-1 to unprimed syntaxin 1A is already known to occur, binding to the primed state has not previously been demonstrated. Sec-1- related proteins are essential to secretion in a number of organisms and

Abbreviations used: DBH, dopamine β -hydroxylase; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EGS, ethyleneglycol-bis-(sulphosuccinimidylsuccinate); GST, glutathione S-transferase; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNAP-25, 25 kDa synaptosome-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor; VAMP, vesicle-associated membrane protein.

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are conserved from yeast to mammals but their precise role remains largely speculative [13]. We also examined the association of these two proteins in more complex settings using both permeabilized chromaffin cells and isolated chromaffin-granule membranes. The results of this portion of the study suggest that more than 50 % of the nSec-1 localized to chromaffin granules is in a dimeric complex with syntaxin 1A, strengthening the argument that this interaction is of physiological significance. The regulation of nSec-1 binding to syntaxin 1A may be important in controlling the availability of syntaxin for participation in SNARE complex formation between vesicles and plasma membrane that is required for membrane fusion.

MATERIALS AND METHODS

Recombinant proteins

A plasmid encoding the cytoplasmic domain of syntaxin 1A (residues 4–266) fused to glutathione S-transferase (GST) was a gift from Dr R. Scheller (Howard Hughes Medical Institute, Stanford University, CA, U.S.A.). An N-terminally truncated syntaxin (residues 195–263) was generated from this construct [10]. Polyhistidine-tagged NSF, α -SNAP, and nSec-1 were prepared as described previously [14,15]. A plasmid encoding the cytoplasmic domain of VAMP (residues 1–86) fused to GST was a kind gift from Dr P. Washbourne (Centro CNR Biomembrane, Università di Padova, Padua, Italy). The GST–VAMP construct was used to transform M15 [pREP4] cells (Qiagen) and protein expression was induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside for 4 h. Protein was purified from cytosolic fractions on glutathione–Sephacrose 4B (GST–Sephacrose, Pharmacia) and the GST moiety cleaved away by incubation with 8 units of thrombin (7500 units/mg, Pharmacia) for 12 h at 4 °C.

VAMP disassembly assay

GST–syntaxin^{4–266} was immobilized on to 20 μ l of GST–Sephacrose at a concentration of 1 μ M by mixing for 30 min at 4 °C. The cytoplasmic domain of VAMP (1 μ M) was added to the incubations and mixed for 1 h at 4 °C. α -SNAP was then added at a concentration of 5 μ M and the incubations continued for a further 1 h at 4 °C with mixing. The beads were pelleted and the supernatants were removed. For primed incubations NSF was added in 100 μ l of binding buffer [20 mM Hepes, 150 mM NaCl, 1 mM dithiothreitol, 2 mM MgCl₂, 1 mM ATP, 0.5 % (v/v) Triton X-100, pH 7.4] at a concentration of 0.1 μ M. Unprimed incubations contained NSF to the same concentration but in binding buffer containing 5 mM EDTA. Priming reactions were allowed to proceed for 1 h at 4 °C with periodic agitation. Sepharose beads were then pelleted and washed twice with 800 μ l of the appropriate binding buffer (+/– EDTA) prior to transfer to clean Eppendorf tubes. Bead pellets were solubilized in 40 μ l of SDS dissociation buffer [4 % (w/v) SDS, 2 mM EDTA, 10 % (w/v) sucrose, 1 % (v/v) β -mercaptoethanol, 10 % (v/v) glycerol, 125 mM Hepes, pH 6.8] and boiled for 5 min. Samples were analysed by SDS/PAGE [15 % (w/v) gel] and transferred to nitrocellulose, by transverse electrophoresis, for immunoblotting. NSF and α -SNAP were detected using anti-polyhistidine antibodies (1:3000, Sigma), VAMP was detected with a guinea-pig polyclonal antibody [16]. All proteins were made visible using enhanced chemiluminescence reagents (Amersham) and digital gel documentation (UVP).

Syntaxin priming and trypsinization assay

GST–syntaxin^{4–266} (5 μ M), α -SNAP (5 μ M) and NSF (0.1 μ M) were mixed in 50 μ l binding buffer (primed) or binding buffer

containing adenosine 5'-[γ -thio]-triphosphate (ATP [S]) in place of ATP (unprimed) and incubated for 2 h at 4 °C. Trypsin (Sigma) was then added to samples at a concentration of 60 nM and proteolysis allowed to proceed for 15 min at room temperature. Digestions were arrested by the addition of 50 μ l of SDS dissociation buffer and immediate boiling. Samples were run on 15 % polyacrylamide gels and transferred to nitrocellulose for immunoblotting. Blots were probed with mouse monoclonal anti-syntaxin antibodies (HPC-1 clone, Sigma) at a 1:500 dilution.

Analysis of rat brain protein binding to syntaxin 1A

Rat brain cytosolic and membrane protein fractions were prepared as described previously [17]. For primed/unprimed reactions, syntaxin was immobilized on to 20 μ l of GST–Sephacrose at a concentration of 2 μ M in binding buffer by incubation for 30 min at 4 °C with constant agitation. α -SNAP and NSF were added to primed incubations at 5 μ M and 0.1 μ M respectively. Control samples contained only GST–Sephacrose and binding buffer to the same final volume as for primed reactions. Incubations were then continued for a further 2 h at 4 °C with occasional mixing. Bead pellets were washed twice in 800 μ l of binding buffer and once in 800 μ l of wash buffer (25 mM Tris/HCl, 50 mM KCl, 1 mM dithiothreitol, pH 7.8). A 250 μ l aliquot of either cytosol or membrane protein was then added to the appropriate incubation and left mixing for 30 min at 4 °C. Pellets were washed three times in 500 μ l of wash buffer (cytosolic samples) or wash buffer plus 1 % (w/v) CHAPS (membrane protein samples). Beads were recovered by centrifugation (10000 g, 1 min) extracted in 50 μ l of SDS dissociation buffer then boiled for 5 min. Protein samples were analysed by SDS/PAGE (12.5 % gel) followed by silver staining or immunoblotting with anti-nSec-1 (1:250, Transduction Laboratories).

Recombinant nSec-1/syntaxin 1A/syntaxin^{195–263} binding assays

GST–syntaxin^{4–266} or GST–syntaxin^{195–263} (both at 1 μ M) were immobilized on to 20 μ l of GST–Sephacrose by incubation for 30 min at 4 °C with mixing. The beads were pelleted and the supernatants were removed. Recombinant His₆-nSec-1 (1 μ M) was then added to all incubations and allowed to bind syntaxins for 5 min. Beads were washed three times with 800 μ l of binding buffer, solubilized in 40 μ l of SDS dissociation buffer and then boiled for 5 min. Proteins were analysed by SDS/PAGE (12.5 % gel) and silver staining.

Subcellular fractionation of adrenal medulla

For the preparation of chromaffin-granule membranes from a large-granule fraction [18], bovine adrenal medullas were dissected and homogenized in buffer A (0.3 M sucrose, 5 mM EDTA, 5 mM Hepes, pH 7.3), and a post-nuclear supernatant prepared and fractionated by differential centrifugation as described previously [19]. For the fractionation of post-nuclear supernatant on sucrose gradients, the post-nuclear supernatant was centrifuged at 100000 g for 60 min, and the pelleted organelles were then resuspended in buffer A, loaded on a 0.3–2.0 M linear sucrose gradient and centrifuged at 100000 g for 90 min. Fourteen fractions of 1 ml were collected for analysis. For the fractionation of the lysed granule [19], a large-granule fraction was lysed by resuspension in 5 mM EDTA, 20 mM Hepes, pH 7.3, followed by a cycle of freezing and thawing. After washing, the membranes were loaded on to a 0.3–1.2 M linear sucrose gradient, centrifuged at 115000 g for 90 min and 14 fractions of 1 ml were collected. Fractions were analysed by

SDS/PAGE (12.5 % gel) and transferred to nitrocellulose. Filters were probed with monoclonal anti-syntaxin antibody (1:500), anti-nSec-1 antibody (1:250), anti-(dopamine β -hydroxylase) (DBH) antibody (1:200; a gift from Dr D. Apps, University of Edinburgh, U.K.) or anti-(Na/K ATPase) antibody (1:500, Upstate Biotechnology).

Granule membrane crosslinking assays

Granule membranes (20 μ l, approx. 37.5 μ g protein) were incubated with various chemical crosslinking reagents, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC; Sigma) and water soluble ethyleneglycol-bis-(sulphosuccinimidylsuccinate) (EGS; Calbiochem), all used at 10 mM, for 30 min at room temperature. All crosslinking reagents were dissolved in granule buffer (20 mM Hepes, 5 mM EDTA, pH 7.4). A further 400 μ l of granule buffer was then added to each incubation and the membranes pelleted (10000 g, 5 min). Supernatants were removed and membranes solubilized in 80 μ l of SDS dissociation buffer +/– boiling for 5 min. Samples were analysed by SDS/PAGE (7.5 % gel) and transferred on to nitrocellulose membranes. Immunoblots were probed with either anti-syntaxin antibody (1:500) or anti-nSec-1 antibody (1:250).

RESULTS

We used an *in vitro* priming reaction to study the properties of recombinant syntaxin 1A in comparisons between primed versus unprimed states. As a confirmation of the validity of our priming system we used, as a marker, the inability of recombinant VAMP to re-associate with syntaxin 1A after ATP hydrolysis had been allowed to proceed in the presence of NSF/ α -SNAP (Figure 1). Complete disassembly of VAMP from syntaxin 1A was reproducibly observed following priming. A second marker for primed syntaxin 1A inherent to this assay is the inability of α -SNAP to remain bound in the presence of NSF and hydrolysable ATP. A small amount of NSF apparently remained bound in this assay, but in others it was completely dissociated (see below).

The binding characteristics of primed syntaxin 1A are distinct from those of the unprimed protein and it has been suggested that priming involves a major refolding of the protein [9]. In an attempt to provide evidence supporting a conformational change, we analysed the sensitivity of primed/unprimed states of syntaxin

1A to the protease trypsin (Figure 2). During initial experiments using only unprimed GST-tagged syntaxin 1A, we observed a number of proteolytic fragments on exposure to trypsin. The first fragment generated was approx. 29 kDa, which, in time course experiments (results not shown), was rapidly degraded to a low molecular mass (< 10 kDa) band. For subsequent experiments, trypsin was used at a concentration and time where all of the GST-syntaxin 1A was cleaved to a single immunoreactive < 10 kDa band under control conditions (Figure 2, lanes 5–8). When priming conditions were imposed (Figure 2, lanes 1 and 2), the sensitivity to trypsin was reduced and the 29 kDa band was protected. The protection of the 29 kDa band was not due simply to complex formation with α -SNAP and NSF, since under conditions where ATP hydrolysis could not occur and the complex could not disassemble, less of the 29 kDa band was present (Figure 2, lanes 3 and 4). The increase in the 29 kDa band was therefore a specific consequence of priming.

Since priming of syntaxin 1A results in a conformational change, it is conceivable that this would permit syntaxin 1A to bind alternative proteins. We aimed therefore to look for any new protein–protein interactions of primed syntaxin 1A. Initial experiments used rat brain membrane or cytosol protein fractions that were incubated with GST-syntaxin 1A under priming conditions where recombinant VAMP and α -SNAP had been shown to dissociate (Figure 1). In comparison with bead controls (i.e. without bound syntaxin 1A), the only specific binding partner from membrane or cytosol fractions under all the conditions was a band of approx. 66 kDa. Figure 3(A) shows a region of the gel to highlight this band which reproducibly bound under both primed and unprimed conditions with similar stoichiometry. Since nSec-1 is known to associate specifically with syntaxin 1A [20] and has a defined molecular mass of 67 kDa, we proceeded to determine whether the syntaxin 1A interacting protein from rat brain extracts was nSec-1. Immunoblotting confirmed the presence of nSec-1 bound to syntaxin from both cytosolic and membrane fractions (Figure 3B).

In order to rule out the possibility that nSec-1 binding following priming could be due to factors in the cytosol or membrane reversing the primed state, we determined whether it was possible to replicate these findings with isolated recombinant proteins. GST-syntaxin 1A was incubated with recombinant nSec-1 when in its native, unprimed, state or following a priming reaction (+ NSF, α -SNAP and MgATP). Under both conditions, similar amounts of the nSec-1 protein were observed to bind to syntaxin 1A (Figure 4A, lanes 5–8). In this experiment a control priming reaction highlighting complete disassembly of NSF from the syntaxin was also included (Figure 4A, lanes 1–4). No binding of nSec-1 was observed in control incubations with GST-Sepharese alone (results not shown). It therefore appears that the initial data suggesting an interaction of nSec-1 with both primed and unprimed forms of syntaxin 1A, are reproducible in a minimal system containing only nSec-1, syntaxin 1A and the priming factors NSF and α -SNAP. As a further indication of the specificity of the nSec-1/syntaxin 1A interaction, a binding assay using both the full cytoplasmic domain of syntaxin 1A (residues 4–266) and an N-terminally truncated mutant of the protein (residues 195–263) with recombinant nSec-1 was performed. This specificity check (Figure 4B) confirmed that only the full cytoplasmic domain of syntaxin 1A bound nSec-1.

Although the nSec-1/syntaxin 1A complex has been well characterized *in vitro* [20,21], little is known concerning the association of these two proteins in more intricate cellular settings, and so we probed the nature of this interaction further. The initial part of this analysis set out to more accurately characterize the cellular distributions of the two proteins in order

Figure 1 Disassembly of VAMP from syntaxin 1A

GST-syntaxin 1A was pre-incubated with the cytoplasmic domain of VAMP. One set of incubations were then subjected to conditions of priming (NSF/ α -SNAP/MgATP) and a second to conditions preventing priming (NSF/ α -SNAP/MgATP/EDTA). Duplicates of both reactions are presented. As well as complete VAMP disassembly on priming, α -SNAP was also incapable of re-associating with syntaxin 1A. Proteins bound to GST-syntaxin 1A were detected by immunoblotting as described in the Materials and methods section.

Figure 2 Trypsin sensitivity of primed versus unprimed syntaxin 1A

GST-syntaxin 1A was incubated in the presence of NSF and α -SNAP to allow the assembly of a complex under conditions of priming (lanes 1 and 2), or non-priming where the complex cannot disassemble (lanes 3 and 4). The sensitivity of a 29 kDa proteolytic syntaxin 1A fragment to 60 nM trypsin was reduced on priming. Lanes 5–8 show control incubations, that contained only GST-syntaxin 1A in the presence or absence of hydrolysable ATP; the 29 kDa species was not protected under these conditions. Duplicate incubations of each condition are presented. ATP γ S, adenosine 5'-[γ -thio]-triphosphate.

to gain an insight into their likely site of interaction *in vivo*. First we examined the distribution of syntaxin 1A, nSec-1, a chromaffin-granule specific marker, DBH, and a plasma membrane specific marker, Na/K ATPase, in fractions from a post nuclear supernatant of bovine adrenal medullae separated on a 0.3–2.0 M sucrose gradient (Figure 5A). It was apparent from the DBH immunoblots of these fractions that there was enrichment of this protein in the denser region of the gradient (fractions 10–14), the region where intact granules are expected to concentrate. Conversely, the Na/K ATPase protein distributed in a less dense region of the gradient (fractions 3–7). Syntaxin 1A was distributed throughout the gradient but suggested a co-distribution with both plasma membrane and granules. The

Figure 4 Interaction of recombinant nSec-1 with GST-syntaxin 1A

(A) GST-syntaxin 1A was primed with NSF/ α -SNAP/MgATP or used in its native unprimed state. nSec-1 bound to syntaxin 1A with similar efficiency under both primed (lanes 5 and 6) and unprimed conditions (lanes 7 and 8), and the interaction displayed an apparent 1:1 stoichiometry. Controls were also included in the experiment to indicate that priming had occurred. NSF can be observed to completely disassemble from syntaxin 1A after priming (lanes 1 and 2), but remained bound in the presence of EDTA (lanes 3 and 4). (B) Control assay highlighting the requirement of the full cytoplasmic domain of syntaxin 1A for efficient binding to nSec-1. The full length cytoplasmic domain of syntaxin 1A (Synt^{195–266}) or an N-terminally truncated derivative (Synt^{195–263}), both fused to GST, were incubated with recombinant His₆-tagged nSec-1 in duplicate reactions. Only the full length syntaxin 1A protein efficiently bound nSec-1. Note that a polypeptide running between nSec-1 and syntaxin 1A is a proteolytic fragment of recombinant nSec-1 [15].

distribution of nSec-1 was similar but displayed greatest concentration in fractions 3–6, which are enriched for plasma membrane material. As a further refinement of the subcellular

Figure 3 Analysis of rat brain protein-binding to primed and unprimed syntaxin 1A

(A) GST-syntaxin 1A was subjected to the same priming reaction shown to cause disassembly of VAMP and α -SNAP. Unprimed samples were given no NSF or α -SNAP. Both primed/unprimed syntaxins were then incubated with either cytosolic or membrane protein fractions derived from rat brain in duplicate. Silver stained gels highlighted a band of approx. 66 kDa that reproducibly bound to both forms of the syntaxin 1A protein following incubation with cytosolic or membrane protein preparations. (B) Immunoblotting of rat brain protein-binding assay. Samples from the experiment described above were transferred to nitrocellulose and probed with an anti-nSec-1 antibody in order to determine whether the binding partner for primed/unprimed syntaxin 1A from rat brain protein samples was nSec-1. An immunoreactive band was clearly present in both primed and unprimed samples. No nSec-1 binding was observed in control incubations lacking syntaxin 1A.

Figure 5 Distribution of syntaxin 1A and nSec-1 in bovine adrenal medulla fractions

(A) Immunoblotting of post-nuclear adrenal medullae supernatant-fractions for a chromaffin-granule marker (DBH) showed enrichment of granules in the denser region of the gradient (fractions 10–14). Immunoblotting of the same fractions with a plasma-membrane-specific marker (Na/K ATPase) highlighted enrichment for plasma-membrane material in a less dense region of the gradient (fractions 3–7). Syntaxin 1A is detectable in all fractions but showed a partial enrichment in the dense region of the gradient, nSec-1 had a similar distribution but was enriched in a less dense region (fractions 3–6). (B) Distribution of syntaxin 1A and nSec-1 following fractionation of lysed chromaffin granules. Again DBH was used to identify granule membranes which were shifted along the gradient to a position of lower density following lysis. A co-distribution of syntaxin 1A and nSec-1 with the granule-membrane marker was clearly apparent. The Na/K ATPase plasma-membrane marker, when applied to this gradient, generated no visible signal (results not shown).

localization of syntaxin 1A/nSec-1 we took isolated chromaffin granules and subjected them to lysis conditions. This preparation was then centrifuged through a second linear gradient intended to purify granule membranes from contaminating material. On this type of gradient (0.3–1.2 M sucrose), the membranes are shifted in position because of their reduced density compared with intact granules. Immunoblots with the same antisera applied to the first gradient highlight a co-distribution of syntaxin 1A and nSec-1 in the granule membrane containing fractions of the gradient (Figure 5B), suggesting that both syntaxin 1A and nSec-1 are associated with chromaffin-granule membranes. Blots of this gradient with the plasma membrane marker yielded no visible signal in any of the fractions (results not shown).

As an extension to the fractionation analysis, we attempted to identify a *bona fide* interaction between syntaxin 1A and nSec-1. Two model systems were employed during this portion of the study, permeabilized adrenal chromaffin cells and a simplified version consisting of isolated chromaffin-granule membranes.

The experimental approach employed involved the incubation of cells or membranes with various chemical crosslinking reagents, some of which, we surmised, might generate syntaxin 1A/nSec-1 complexes which would then be detectable by immunoblotting. Initial experiments utilizing permeabilized chromaffin cells exhibited a very low efficiency of crosslinking with only a small amount of total syntaxin 1A found in a putative dimeric complex with nSec-1 (results not shown). One possible explanation for the low efficiency of crosslinking observed in these assays could be due to the presence of other proteins and protein complexes that act as alternative and competing targets for the crosslinking reagents. We therefore proceeded to analyse this potential interaction in granule membranes where direct access of the crosslinker to the protein on the cytoplasmic surface of the membrane would be possible. In this series of experiments, we were able to reproducibly and efficiently detect a putative syntaxin 1A/nSec-1 complex (Figure 6). A number of crosslinking reagents were employed, having variable sized spacer regions between the

Figure 6 Chemical crosslinking of isolated chromaffin-granule membranes and detection of a putative dimeric nSec-1/syntaxin 1A complex

Chromaffin-granule membranes were incubated with or without the indicated crosslinking reagents (EDAC and EGS) at 10 mM for 30 min at room temperature. Membranes were then pelleted and solubilized in SDS sample buffer for analysis by SDS/PAGE. Samples were transferred to nitrocellulose filters for immunoblotting with either anti-syntaxin 1A (A) or anti-nSec-1 (B) antisera. Only the EDAC reagent reproducibly generated a high molecular mass complex (approx. 100–110 kDa) which appeared on blots for both syntaxin 1A and nSec-1 (triangle). Generation of this complex was specific as it failed to appear under any other conditions and was distinct from other high molecular mass species such as those present in samples of unboiled granule membranes representing the assembled SNARE complex and its oligomers.

functional groups, however only the reagent with the shortest spacer arm, EDAC, but not EGS for example, gave rise to an adduct that appeared in both syntaxin 1A and nSec-1 immunoblots. The band which appeared only following incubation of membranes with EDAC corresponded to a molecular mass of approx. 100–110 kDa, similar to the calculated mass of a syntaxin 1A/nSec-1 dimer. Reprobes of syntaxin 1A blots with the nSec-1 antisera gave rise to labelling of the same high molecular mass band, which exactly overlaid on both blots (results not shown). Furthermore, significant amounts of both total syntaxin 1A and nSec-1 in the membrane preparation were detected in the complex. Approx. 54 % of total nSec-1 and 30 % of total syntaxin 1A were present in the dimeric species. The same experiment performed on plasma membrane containing chromaffin cell fractions yielded essentially the same result (results not shown).

The syntaxin 1A immunoblot (Figure 6) also displays samples of non-crosslinked granule membrane, which were either boiled or not boiled in the presence of SDS dissociation buffer. There are a number of bands in the unboiled sample, one at approx. 70 kDa may correspond to the SDS-resistant 7 S SNARE complex [22]. It is interesting to note that none of the bands observed in the unboiled membrane sample appear in the EDAC crosslinking track, highlighting the fact that formation of the putative syntaxin 1A/nSec-1 dimeric species is highly specific. These results provide evidence for an nSec-1/syntaxin 1A interaction on the surface of chromaffin-granule membranes.

DISCUSSION

The t-SNARE syntaxin 1A [23] has emerged as a protein of central importance to the process of regulated exocytosis in a number of neuronal cell types [24]. Observations that syntaxin 1A along with its partner SNAREs, VAMP and SNAP-25, are the targets for the potent clostridial neurotoxins [25], supports an acute role for these proteins in regulated secretory events. Syntaxin 1A is of particular interest because of its ability to

interact with a large variety of other exocytotic proteins [26–28]. Unlike VAMP and SNAP-25, syntaxin associates with multiple protein partners, which may represent interactions specific to distinct steps in the vesicle–plasma membrane fusion cycle.

The most intensively characterized syntaxin interaction concerns its role in the so-called SNARE complex [8], a process initially believed to underpin actual membrane fusion events. In recent years a significant body of data has accumulated from both *in vitro* and *in vivo* sources to question this view of SNARE function, with one such observation relating to an unusual state conferred on the syntaxin protein during disassembly of the SNARE complex. This so-called ‘priming’ reaction is interesting in that it dramatically alters the binding properties of syntaxin with respect to its normal protein partners [9]. Priming of the t-SNARE homologue is a prerequisite to fusion in a yeast vacuole fusion system [12] and permits binding of the t-SNARE to a new protein partner LMA1 [29]. It has been shown that primed syntaxin 1A is no longer able to bind either VAMP or α -SNAP and we were therefore interested to ascertain whether this state simply acts to sequester syntaxin from vesicle cycling reactions, or whether it serves some alternative purpose.

First, we aimed to establish a syntaxin 1A priming assay for use in further experiments. We have demonstrated NSF ATPase activation in a minimal system containing only syntaxin 1A previously [10], and were therefore able to apply this simplified model to the analysis of priming. Our results highlighting VAMP/ α -SNAP dissociation from syntaxin, as markers for priming having successfully occurred, demonstrate the validity of this assay system. To provide corroborating evidence for priming involving a conformational switch in the syntaxin 1A protein, we also examined the protease sensitivity of syntaxin that had experienced the priming assay and compared this to control, unprimed syntaxin. These experiments highlighted a significant decrease in the protease sensitivity of syntaxin 1A on priming, that was not due simply to complex formation with α -SNAP and NSF, suggesting a definite conformational change due to the priming reaction.

If priming simply behaves as a time-dependent sequestration mechanism for syntaxin [9], then immediately after the reaction has occurred, it should be possible to observe an inert state of the protein that is unable to mediate normal binding responses. We tested this theory *in vitro*, in assays where primed and unprimed syntaxins were incubated with rat brain cytosol and membrane protein samples. We observed the reproducible binding of an approx. 66 kDa band to syntaxin, under both primed and unprimed conditions, and from both brain fractions, which we were subsequently able to identify as nSec-1 by immunoblotting. This result is intriguing since it suggests that the priming-induced conformational alteration in syntaxin maintains binding to nSec-1 but not to VAMP or α -SNAP. This result is rendered all the more surprising in view of the fact that nSec-1 apparently requires the entire cytoplasmic domain of syntaxin 1A for efficient association [20]. The N- and C-terminal domains may not both bind directly to nSec-1 but they may contribute towards maintaining the necessary folding of the protein. The interaction was confirmed with recombinant, bacterially expressed syntaxin 1A and nSec-1 *in vitro*, proving that the association isolated in a complex protein mixture was reproducible in a minimal system, and that other factors present in the cytosol and membrane fractions had not simply reversed the primed state of the syntaxin. The fact that primed syntaxin 1A is able to interact with at least one of its normal protein partners argues against this reaction as an inactivation mechanism and rather suggests that the nSec-1 protein may have potential roles in post priming functions of syntaxin 1A.

The exact function of nSec-1 in neuronal secretion, and its homologues at other trafficking steps in various organisms, is presently unclear. The first member of this protein family, Sec1p, was isolated as a yeast temperature-sensitive mutant defective in a terminal step of exocytosis [30]. Many Sec1-like proteins have since been discovered in numerous organisms and it now seems apparent that specific vesicular transport steps within a given cell type utilize specific Sec-1-related proteins [13]. Although the role of these proteins is currently speculative, the severe phenotypes induced by mutations in their genes argues for a critical role in secretory processes. Current evidence is divided as to the exact nature of Sec1-like protein function. A positive effect is supported in studies where mutant phenotypes exhibit accumulation of transport vesicles, hinting at defective vesicle docking and/or fusion [30–32]. Other studies, however, provide evidence for a negative function [33], and it now appears that Sec-1-related proteins are likely to effect both positive and negative regulatory functions [34,35].

The interaction of the mammalian neuronal Sec-1 homologue, nSec-1, with syntaxin 1A has been well characterized *in vitro* [20,21] but only for the non-primed state. In these studies it was shown that syntaxin 1A and nSec-1 bind with high affinity, and further functional studies provided evidence that this is a critical interaction *in vivo* [36,37]. Isolation of the nSec-1/syntaxin 1A complex formed *in vivo* has, however, proven difficult [38]. In cellular settings, nSec-1 distributes into both cytoplasmic and membrane-bound pools, yet it is essentially a hydrophilic protein and consequently is likely to require a membrane receptor for localization to these sites. The obvious candidate for the membrane receptor is syntaxin; however, in sedimentation studies using rat brain post-nuclear supernatants it appeared that nSec-1 was not in a heteromeric complex with syntaxin 1A [38]. It seems unlikely, based on functional studies, that syntaxin and nSec-1 are not associated within cells to some degree and we were interested to extend the initial analysis of priming to look at the distribution and association of these proteins in bovine adrenal chromaffin cells. We have been able to demonstrate a co-distribution of a proportion of total cellular nSec-1 and syntaxin 1A with a chromaffin-granule specific marker, a result which agrees with and extends published observations concerning the presence of syntaxin 1A on chromaffin granules [39]. The presence or absence of nSec-1 on dense-core granules has, however, been controversial [38,40]. Previous work on nSec-1 showed the protein to be present in a crude granule membrane fraction but no data describing the distribution in gradient fractionation were shown. In contrast, other studies have failed to localize nSec-1 to secretory granules [38]; however these experiments employed PC12 cells with relatively low numbers of secretory granules, compared with chromaffin cells, and as a result the presence of nSec-1 may have been difficult to observe.

In order to further our understanding of nSec-1/syntaxin 1A interactions we have also been able to successfully demonstrate a putative dimeric complex of the two proteins in plasma membrane-enriched and chromaffin-granule membrane preparations. Using a chemical-crosslinking strategy we have been able to show that a significant proportion of both total granule-associated nSec-1 (30%) and syntaxin 1A (54%) are present in the putative complex. Similar experiments with plasma membrane-enriched material yielded similar results.

Current models of nSec-1 function in regulated secretion remain vague; however, one point seems clear, that the interaction of nSec-1 with syntaxin 1A is a critical one in the overall scheme of vesicle/membrane trafficking. Until now, functional studies have strongly advocated this view, however proof of a physical interaction between these two proteins *in vivo*

has remained elusive. Our data for the first time provide evidence for such a physical association and hint at a possible connection between the process of SNARE priming and an increase in nSec-1/syntaxin 1A complex formation. These results also point to a potentially important role for nSec-1/syntaxin 1A interactions on the secretory granule. While it is clear that association of syntaxin 1A in the SNARE complex is controlled by α -SNAP and NSF [8], the regulation of nSec-1 association with syntaxin 1A remains to be understood. Disassembly of a 7 S complex and priming of syntaxin 1A may occur on undocked chromaffin granules [41]. The present results also raise the possibility that association of nSec-1 with syntaxin 1A, and its regulation, on secretory vesicles as well as the plasma membrane, may be physiologically significant for the steps leading to vesicle tethering, docking and fusion. In particular, the ability of nSec-1 to bind to syntaxin 1A in both the primed and unprimed states suggests that nSec-1 can act as a chaperone-like partner for syntaxin 1A throughout its functional cycle. This will also mean that control of the disassembly of the dimeric complex, perhaps via a rab effector [34,42], will be crucial in the control of free syntaxin 1A availability for its participation in the SNARE complex immediately prior to membrane fusion. Further work will be required to resolve the mechanism involved in this regulation.

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