Protein-binding partners of the tobacco syntaxin NtSyr1

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Abstract Syntaxins and other SNARE (soluble NSF-attachment protein receptor) complex proteins play a key role in the cellular processes of vesicle trafficking, vesicle fusion and secretion. Intriguingly, the SNARE NtSyr1 (= NtSyp121) from *Nicotiana tabacum* also appears to have a role in signalling evoked by the plant stress hormone abscisic acid. However, partner proteins contributing to its function(s) remain unknown. We used an affinity chromatography approach to identify proteins from tobacco leaf microsomes that directly interact with the hydrophilic (cytosolic) domains of NtSyr1 and report several interacting proteins with sensitivities to the endopeptidase activity of *Clostridium botulinum* neurotoxins, including one protein that was recognised by αAtSNAP33 antiserum, raised against the *Arabidopsis* SNAP25 homologue. Treatment of microsomal membrane fractions indicated a protein near 55 kDa was sensitive to proteolysis by BotN/A and BotN/E, yielding degradation products of approximately 34 and 23 kDa. Expressed and purified AtSNAP33 also bound directly to the cytosolic domain of NtSyr1 and was sensitive to proteolysis by these toxins, suggesting that NtSyr1, a tobacco homologue of AtSNAP33, and coordinate SNAREs are likely to associate as partners for function in vivo.

Key words: Plant plasma membrane secretion; Affinity chromatography; Botulinum neurotoxin; SNARE; Abscisic acid

1. Introduction

The underlying molecular mechanisms of intracellular vesicle traffic and membrane fusion appear to be conserved amongst eukaryotic organisms, from yeast to mammals and plants [1] and depend on SNARE (soluble NSF-attachment protein receptor) proteins as well as regulatory factors, including nSec1, Rab GTPases, α-SNAP and NSF [2]. The so-called SNARE hypothesis proposes that selectivity of membrane fusion is achieved by the interaction of t-SNAREs (target membrane SNARE) located on the cytosolic surface of the target membrane, and v-SNAREs (donor vesicle SNAREs) present on the cytosolic surface of the cargo vesicles [3–5]. In presynaptic nerve terminals, the t-SNAREs syntaxin 1 and SNAP25 (synaptosome-associated protein of 25 kDa) together with the v-SNARE VAMP/synaptobrevin 1 form a ternary complex through hydrophobic interactions of coiled-coil domains [5–7].

A number of plant SNARE proteins have been isolated both by functional complementation of corresponding mutations in yeast (see [1] for review) and, recently, through the *Arabidopsis* genome sequencing project [8]. With few exceptions [9,10] their functions remain to be demonstrated in planta. Nonetheless, many of these proteins exhibit a high degree of conservation with their yeast and mammalian counterparts, implying similar roles within the cell.

The NtSyr1 protein (= NtSyp121 [8]) from *Nicotiana tabacum* was the first plasma membrane-localised syntaxin to be identified in plants [11,12]. Similarity of the NtSyr1 amino acid sequence to yeast and mammalian syntaxins suggests the role of the protein in vesicle trafficking to the plasma membrane and recent data support this idea [13]. Nonetheless, an intriguing feature of this protein is its apparent role additionally in signalling events evoked by the plant stress hormone abscisic acid (ABA) [11,12].

Because of the potential juxtaposition of role(s) for NtSyr1, it will be important to identify proteins that interact with NtSyr1 and to characterise their activities. As a first step, we have developed an affinity chromatography approach to purify several membrane-associated partners that interact directly with the hydrophilic (cytosolic) domains of NtSyr1. Here we describe the method, and demonstrate that one of the affinity-purified NtSyr1-binding partners is likely to represent a tobacco homologue of SNAP25. We also show that several NtSyr1-interacting partners undergo specific cleavage by clostridial neurotoxins implying their structural similarity to mammalian SNARE counterparts.

2. Materials and methods

2.1. Purification of Sp2 and Sp3 peptides

cDNA fragments corresponding to the Sp2 and Sp3 peptides (His_6-tagged C-terminal truncations of NtSyr1 [11,12]) were cloned into the pQE-30 vector (Qiagen), expressed and purified using QIAexpressionist kit (Qiagen) following the manufacturer's instructions.

2.2. Isolation of AtSNAP33 cDNA, αAtSNAP33 antibody production and Western blot analysis

Expressed sequence tags (ESTs) were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, USA). Full-length cDNAs were isolated by stringent screening of the *Arabidopsis thaliana* PRL2 cDNA library (ABRC) [14], phage propagated and amplified in *Escherichia coli* Y1090ZL (Gibco BRL Life Technologies). Excision of the pZL1-derived plasmids was performed in *E. coli* strain DH10BZIP (Gibco BRL Life Technologies) after plasmid purification (Nucleobond AX100, Macherey-Nagel, Düren, Germany). cDNAs were sequenced using a Sequenase kit (Amersham) or by auto-sequencer (Eurogentech, Seraing, Belgium).

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For the production of antibodies, the *Sal*I (blunt-ended)–*Hin*dIII fragment of the EST accession number T76564 (amino acids 124–300 of the AtSNAP33 protein) was fused in frame between *Bam*HI (blunt-ended) and *Hin*dIII restriction sites to the His_6 tag of plasmid pQE-31 (Qiagen), transformed in *E. coli* M15, and the fusion protein expressed, extracted under denaturing conditions and purified on Ni^{2+}-NTA resin (Qiagen). The His_6-tagged protein was further purified by SDS–PAGE, protein recovered by electroelution (Biotrap, Schleicher and Schuell) and washed with Tris-buffered saline (TBS: 150 mM NaCl, 10 mM Tris–HCl, pH 7.4) before concentrating (Centricon P10, Amicon). Rabbit antisera were obtained following standard procedures [15].

Proteins were analysed by Western blot after electroblotting onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked in TBS-T (TBS, 0.1% Tween-20, 5% semi-skimmed milk) for 2 h and probed overnight at 25°C with primary antibodies diluted 1:3000 in the same buffer. Blots were visualised using ECL™ detection (Amersham) after secondary antibody labelling (1 h, 25°C) with horseradish peroxidase-labelled anti-rabbit antibody (Sigma), diluted 1:25 000 in TBS-T.

2.3. His_6-AtSNAP33 production

The *Msp*I (blunt-ended)–*Hin*dIII cDNA fragment corresponding to amino acids 8–300 of the *AtSNAP33* cDNA (X92420) was fused in frame as described above. The pQE His_6-AtSNAP33 plasmid was transformed into *E. coli* M15, and the protein was expressed and purified under non-denaturing conditions (Qiagen) using Talon resin (Clontech).

2.4. Preparation of microsomal membranes

Mature leaves of 5–6 week old *N. tabacum* were ground in liquid nitrogen and homogenised in 0.5 M sucrose, 50 mM HEPES–NaOH, pH 7.4, 0.1% (w/v) sodium ascorbate, 1 mM EDTA, and Complete™ Protease Inhibitor cocktail (Boehringer). The homogenate was filtered through four layers of Miracloth, centrifuged at $10\,000 \times g$ (10 min, 4°C), the supernatant centrifuged at $50\,000 \times g$ (40 min, 4°C), and the microsomes resuspended in HBS buffer (150 mM NaCl, 25 mM HEPES–NaOH, pH 7.4) supplemented with Complete™ Protease Inhibitor. Aliquots were snap-frozen in liquid nitrogen, and stored at −20°C.

2.5. Affinity chromatography

Sp2 or Sp3 peptide (8–10 mg) was covalently coupled to CNBr-activated Sepharose 4B support (Pharmacia) using carbonate buffer (0.5 M NaCl, 0.1 M $NaHCO_3$, pH 9) and the matrix (1 ml) packed into a C10/10 (Pharmacia). Microsomes were solubilised in 2% CHAPS, 0.2 M phosphate, 0.15 M KCl and 2 mM $MgCl_2$ (3 h, 4°C) and insoluble material removed by centrifugation ($17\,800 \times g$, 33 min, 4°C). Solubilised proteins (10 mg) were loaded onto affinity columns and washed with 150 column volumes of HBS buffer. Bound interactors were eluted with 0.1–1.5 M KCl step gradients in HBS with 0.5% Lubrol-PX, desalted on Econo-Pak 10 DG columns (Bio-Rad), and 1 ml aliquots concentrated by DOC/TCA/acetone precipitation. The bulk of each fraction was concentrated by centrifugation (Centriplus, Amicon). Peptide-specific elution was carried out using 10 μM Sp2 or Sp3 peptide in 0.03 M NaCl with 0.1 M Na-phosphate buffer, pH 8.5. Fractions was exchanged against 10 mM imidazole and 0.8 M NaCl in the same buffer to release complexed proteins and the peptides were removed by passage over Ni^{2+}-NTA (Qiagen) before precipitation as above. The proteins were resolved under reducing conditions by SDS–discontinuous Laemmli PAGE (12.5% polyacrylamide) and visualised by silver staining.

2.6. Neurotoxins

Recombinant vectors pBN3BotNT/A, pBN27BotNT/C, pBN31BotNT/D and pBN17BotNT/E expressing His_6-tagged light chains of corresponding *Clostridium botulinum* neurotoxins were kindly provided by Dr Thomas Binz (University of Hannover) and were expressed and purified as described [16]. Aliquots (1–2 μg protein) of affinity-purified fractions were diluted in HBS buffer with 2 mM MgATP, 0.3 mM $ZnSO_4$, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$ and 2 mM phenylmethylsulphonyl fluoride. Control experiments were carried out without $ZnSO_4$ and with 1 mM EDTA (not shown). Following pre-incubation of fractions (30 min, 30°C) purified neurotoxin light chains were added (BotN/A, 33 nM; BotN/C, 197 nM; BotN/D, 111 nM; BotN/E, 55 nM) and the mixtures incubated for 4 h at 37°C. Proteins were precipitated (DOC/TCA/acetone) and resolved under reducing conditions by 5–20% gradient SDS–PAGE and silver-stained. Optical densities of protein bands in each lane were analysed using ImageMaster 1D software (Pharmacia) following background subtraction.

3. Results and discussion

3.1. Purification of NtSyr1 protein partners

NtSyr1 shares structural features that are common to eukaryotic syntaxin proteins, including a C-terminal transmembrane spanning domain, the highly conserved syntaxin signature sequence within the H3 domain [11,12] that contributes to SNARE complex formation in mammals and yeast [2], and three coiled-coil domains (HA, HB and HC) [1] that in the mammalian counterparts are thought to associate with the H3 region as part of the mechanism regulating SNARE complex formation [6,7]. The so-called Sp2 fragment – corresponding to the entire cytosolic portion of NtSyr1 – was found to interfere in ABA-mediated regulation of K^+ and Cl^- channels [11] and to block secretion at the plasma membrane in vivo [13], suggesting that this fragment competes with the native, full-length NtSyr1 for partners.

To identify putative NtSyr1-interacting partners, we used the Sp2 fragment for affinity chromatography after coupling the fragment to a Sepharose matrix. For comparison, a similar matrix was prepared using a second fragment, Sp3, which comprises the three N-terminal coiled-coil domains (HA, HB and HC), but lacks the syntaxin signature sequence [12]. Fig. 1 shows a typical elution pattern with moderate to high KCl concentrations from Sp2 affinity columns ($n = 8$). In general, some protein, probably incompletely bound, was eluted at 0.1 M KCl, but most was obtained with KCl steps to 0.8 M and above, including prominent bands near 20 kDa, 25 kDa, 29 kDa, 34–36 kDa, 40–42 kDa and 55 kDa (Fig. 1A).

Similar (although not identical, see below) elution patterns were observed from Sp3 affinity columns (Fig. 1B) although, in this case, much of the protein was eluted below 0.5 M KCl. Notably, bands near 40–42 kDa, abundant in 0.8 and 1 M KCl fractions from Sp2 columns, were not clearly resolved from Sp3 columns and bands at 20 and 25 kDa were better resolved on Sp2 affinity columns. Comparable elution patterns were also obtained with Sp2 linked via its N-terminal His_6 tag to an agarose matrix (not shown), and only a trace amount of protein was detected in the fractions eluted with KCl step gradient from the column with covalently bound bovine serum albumin, confirming the ligand specificity and His_6 independence of the protein interactions.

We also eluted interacting proteins using soluble Sp2 and Sp3 fragments, reasoning that these fragments should be most effective in competing off binding proteins with high structural specificity and therefore sharpen the elution profiles (see also [17]). The fragments were removed by passage over Ni^{2+}-NTA in 0.8 M NaCl and 10 mM imidazole to disrupt Sp2 (and Sp3) partner interactions before partner proteins were recovered in 250 mM imidazole. A number of sharply defined membrane protein bands were obtained, ranging in size between 10 and 100 kDa (Fig. 1C, left), similar to those obtained with KCl step gradients. Several novel Sp2-binding partners were also resolved (carets) that were clearly distinguished from possible carry-over of the soluble Sp2 fragment and Sp2 preparation contaminants. A similar, but not identi-

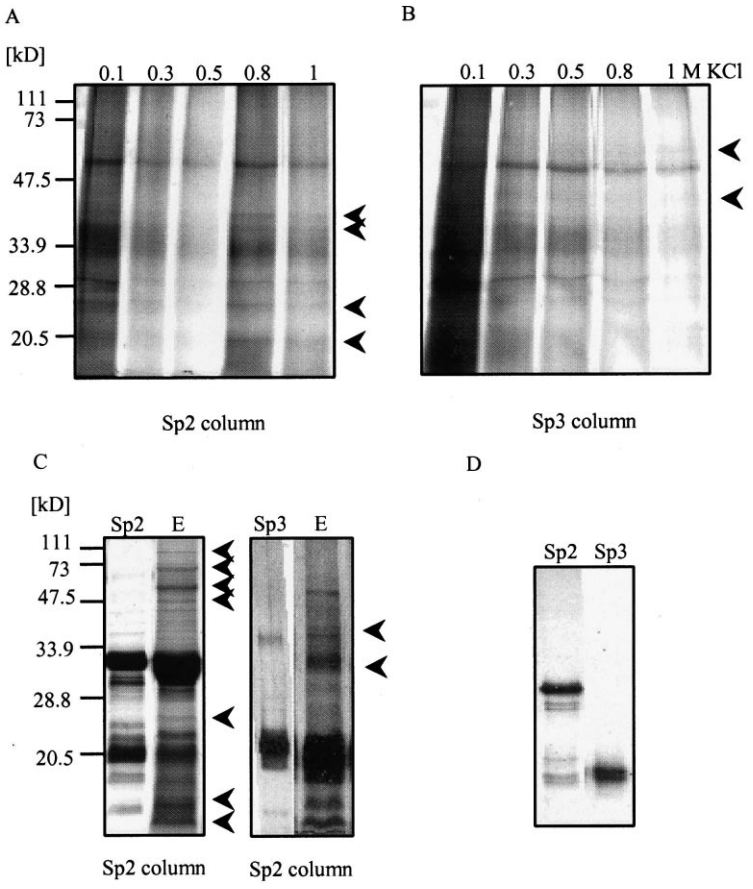

Fig. 1. Affinity purification of proteins interacting with the entire cytosolic domain of NtSyr1 (Sp2) and with the HA/HB/HC subdomain (Sp3). CHAPS-solubilised tobacco microsomal proteins eluted with steps of 0.1–1 M KCl (concentrations, above) from Sp2 (A) and Sp3 columns (B), and with 10 μM soluble Sp2 and Sp3 fragments from an Sp2 column (C). Equal volumes of each fraction (6 μg total protein) were resolved on SDS–PAGE and visualised by silver staining. MW standards indicated (left). Left lanes (Sp2, Sp3), 3 μg cleared fragment; right lanes (E), eluates after clearing of the fragment (original Sp2 or Sp3 content per fraction, 330 μg). Differences in protein profiles distinct from fragment clearing indicated by carets (see text). (D) Silver-stained preparations of Sp2 and Sp3 fragments (0.5 μg protein) used to construct affinity columns.

cal pattern of proteins was obtained on elution with soluble Sp3 fragment (Fig. 1C, right), and an absence of the higher molecular weight bands was evident. Clearing the column first with Sp3, followed by elution with the Sp2 fragment yielded a subset of these bands, the most prominent appearing between approx. 40 and 70 kDa (Fig. 1D, carets), suggesting interactions of these proteins that are specific to the H3 domain of NtSyr1.

3.2. *Cleavage of NtSyr1-interacting partners with botulinum neurotoxins*

As a first step to identifying and characterising the interacting protein fractions, we made use of *C. botulinum* neurotoxins (BotN/*x*, *x* = A–G) that bind and cleave selected SNAREs [2,16,18], including NtSyr1 [11]. Fractions eluted from Sp2 affinity columns were subjected to treatment with purified BotN/A, BotN/C, BotN/D or BotN/E, and the results analysed by SDS–PAGE. Several of the tobacco membrane proteins showed sensitivity to the toxins, especially to BotN/A and BotN/C (see Fig. 2A,B). Protein bands of approximately 22 and 62 kDa were susceptible to BotN/A and BotN/C, respectively, and bands near 18 and 55 kDa were targeted by both toxins and thus may comprise or incorporate t-SNARE (syntaxin and/or SNAP25) homologues of tobacco. Interestingly, a new band appeared at 23 kDa following treatment with BotN/A. This protein may represent a cleavage product of the tobacco SNAP25 homologue (below).

3.3. *Immunochemical analysis of Sp2-interacting proteins*

To date, NtSyr1 is the only SNARE homologue to be cloned from tobacco. Therefore, to test whether NtSyr1 might interact directly with a tobacco homologue of SNAP25, crude microsomal membrane proteins as well as membrane proteins eluted from Sp2 affinity columns were probed with the antiserum generated against the plasma membrane-localised *Arabidopsis* homologue of SNAP25, AtSNAP33 (accession number X92420; Gansel and Sticher, in preparation), and antiserum generated against the Sp3 domain of NtSyr1 [12]. A protein of approximately 55 kDa was the major band identified with αAtSNAP33 antiserum, and was evident in crude tobacco leaf microsomes (TLM), in the CHAPS-soluble fraction and in Sp2 affinity-purified fractions (Fig. 3A). When probed with αSp3 antiserum (Fig. 3B), the same band was detected in addition to cross-reactions with NtSyr1 and a second band around 42 kDa in the crude microsomes and CHAPS-soluble fraction. Both antisera showed a doublet around 55 kDa, albeit with differential sensitivities in the 0.3 M KCl eluate from the Sp2 column.

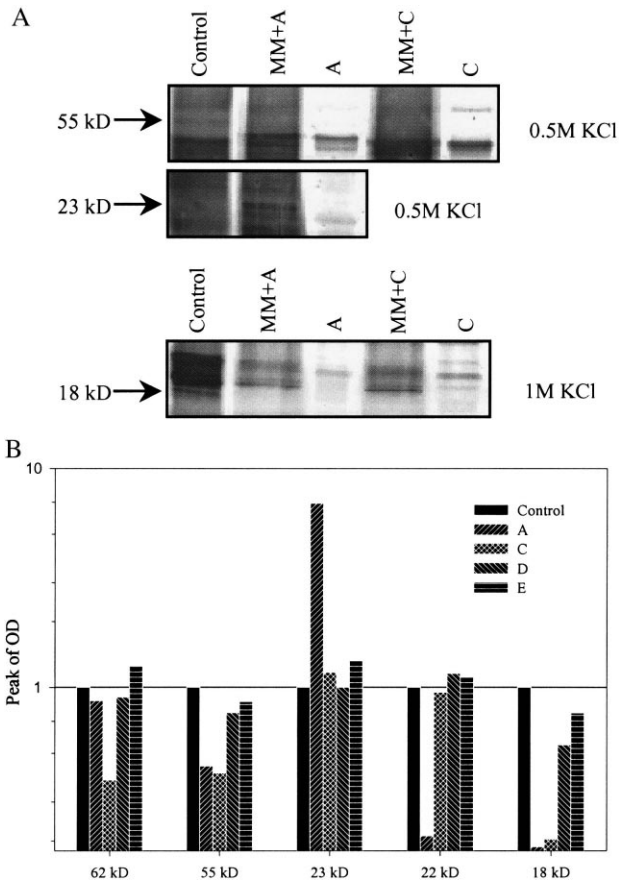

Fig. 2. Botulinum neurotoxins BotN/A, BotN/C and BotN/E target four protein fractions that interact with the cytosolic domain (Sp2) of NtSyr1. Sp2-interacting partners eluted with 0.5 M, 0.8 M and 1 M KCl treated separately with 30–200 nM BotN/A, BotN/C, BotN/D and BotN/E in the presence of 2 mM MgATP (see Section 2). Results of experiments without $ZnSO_4$ and with 1 mM EDTA gave results comparable to controls (not shown). A: Representative silver-stained SDS–PAGE gels (5–20% linear gradient) of CHAPS-solubilised proteins from microsomal membranes (5 μg total microsomal protein) without toxin (Control), with BotN/A (MM+A) and BotN/C (MM+C), and the toxins alone (A and C, 30 and 200 nM, respectively). Segments with three affected bands near 18, 23 and 55 kDa indicated on left. B: Optical densities (OD) of bands integrated using ImageMaster™ software (Pharmacia) and differences ± BotN/*x* (ΔOD) determined after background and BotN/*x* lane subtraction. ΔOD values normalised to the controls (−BotN/x). Note the log scale on the *y*-axis. Molecular sizes of protein bands exhibiting OD changes are indicated below.

That the 55 kDa band was recognised by both αAtSNAP33 and αSp3 antisera could be explained if this fraction incorporated one or more proteins with epitopes recognised by both sets of antibodies. (A single epitope common to both antisera is unlikely, since αAtSNAP33 antibodies did not recognise NtSyr1 (see Fig. 3A).) Alternatively, the 55 kDa band might represent an SDS-resistant complex of protein partners that also bind to the Sp2 fragment of NtSyr1. Binary, SDS-resistant complexes of syntaxin 1 and synaptobrevin/VAMP are known [19], and larger molecular weight protein complexes, equivalent to the canonical 20S core complex of SNAREs [2,5], have been described in preparations with the t-SNARE AtPep12 from *Arabidopsis* [20].

As a first test for the presence of a SNAP25 homologue associated with the 55 kDa protein band, we treated microsomal membranes and Sp2-purified, 50–55 kDa fractions with BotN/*x* neurotoxins. All BotN/*x* toxins alone showed a strongly immunoreactive band near 29 kDa, but no other cross-reactivity with αAtSNAP33. In the absence of BotN/*x* treatment no degradation products were seen (Fig. 4A, left), indicating that cleavage was not the result of endogenous plant proteases. The toxin preparations alone showed cross-reactivity with αAtSNAP33 (compare Fig. 4A and B) with discrete bands near 29 and 50 kDa. Comparison with these controls showed that treatments of microsomal membranes with BotN/A and BotN/E yielded new immunoreactive products in the presence of MgATP (Fig. 4A), notably a prominent band near 23 kDa, close to the position of the low molecular weight product found on silver-stained gels (see Fig. 2), as well as faint bands near 36 kDa. We confirmed the sensitivity of AtSNAP33 specific to BotN/A and BotN/E toxins in vitro, using the His_6-tagged AtSNAP33 purified on Ni^{2+}-NTA (Fig. 4C). Finally, we also examined in vitro the interaction between purified AtSNAP33 and the Sp2 fragment of NtSyr1. His_6-tagged AtSNAP33 was purified on Ni^{2+}-NTA, and then passed through an Sp2 affinity column. Western blot analysis of the KCl-eluted fractions (Fig. 4D) showed that AtSNAP33 bound tightly to the Sp2 ligand and could be

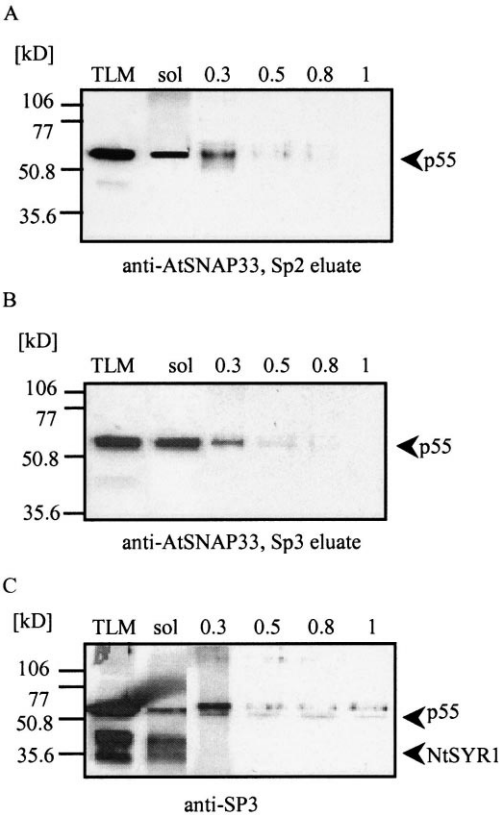

Fig. 3. Antibodies to the tobacco syntaxin NtSyr1 and *Arabidopsis* SNAP25 homologue AtSNAP33 recognise a common 55 kDa protein fraction. Western blots of total TLM proteins and the 2% CHAPS-soluble (sol) fraction after separation by centrifugation. Further separation by passage over Sp2 (A) or Sp3 affinity (B) columns and elution with 0.3–1 M KCl steps yielded the proteins in the subsequent lanes (M KCl concentrations above). In each case, proteins were precipitated, run on 12.5% SDS–PAGE, and probed using either the αAtSNAP33 (A, B) or αSp2 (C) polyclonal antibodies. NtSyr1 and an approx. 55 kDa protein band (p55) indicated by carets (right) and protein size markers in kDa (left).

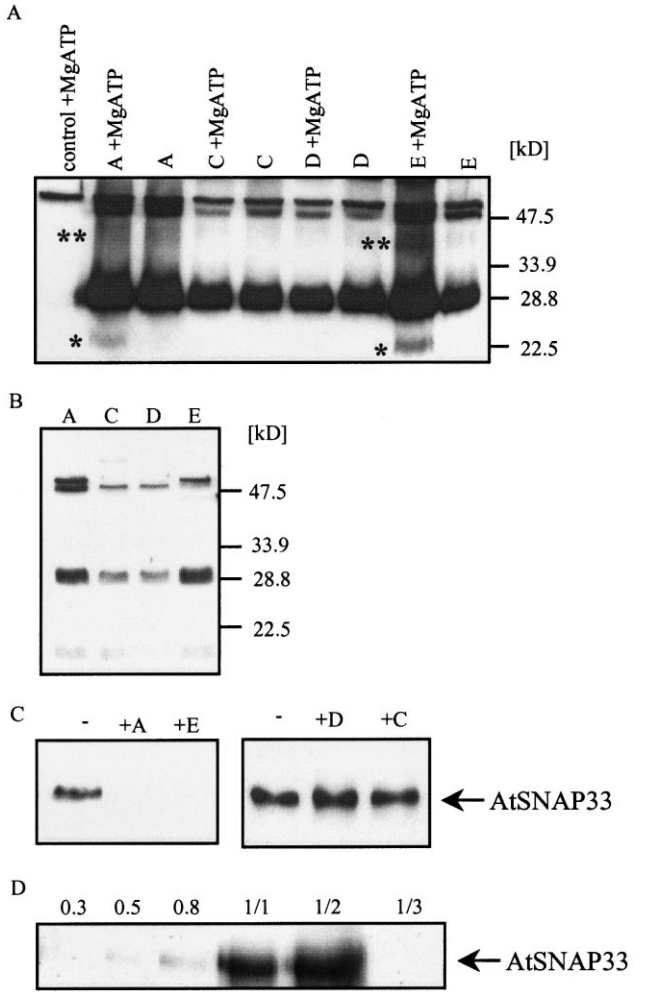

Fig. 4. Botulinum neurotoxins BotN/A and BotN/E specifically target a 55 kDa fraction from tobacco leaf microsomes and the *Arabidopsis* SNAP25 homologue AtSNAP33. A: Western blot analysis of tobacco leaf microsomes (10 μg total protein) treated with BotN/*x* (*x* = A, C, D, E) and ±2 mM MgATP (indicated above each lane). Fractions of the treated mixtures run on 5–20% linear SDS–PAGE gels and probed using αAtSNAP33 polyclonal antibodies. The prominent bands near 29 and 48 kDa are antigenic products within the BotN/*x* preparations and appear also in the absence of all other membrane fractions (below). BotN/A- and BotN/E-specific cleavage products (asterisks) and protein size markers (right). B: Western blot analysis of equivalent aliquots of BotN/*x* as in A resolved by SDS–PAGE and probed with αAtSNAP33 antibody. C: Cleavage of purified His_6-AtSNAP33 (1 μg) in the presence of 2 mM MgATP and BotN/*x* (*x* = A, C, D and E, as indicated above). Proteins separated on 12.5% SDS–PAGE and analysed by Western blotting using αAtSNAP33 polyclonal antibodies. AtSNAP33 cleavage products were not recovered and may be the result of an instability of the partly digested protein [11]. D: In vitro interaction between the cytosolic domain (Sp2) of the tobacco syntaxin NtSyr1 and the *Arabidopsis* SNAP25 homologue AtSNAP33. Purified His_6-AtSNAP33 (0.9 mg) eluted from an Sp2 column with 0.3–1 M KCl steps (M concentrations indicated above lanes), and collected in 6 ml (0.3–0.8 M KCl) and three successive 5 ml (1 M KCl) fractions. One tenth the volume of each fraction was precipitated, separated on 12.5% SDS–PAGE, analysed by Western blotting with αAtSNAP33 polyclonal antibodies.

washed off in appreciable quantities only at KCl concentrations above 0.8 M.

From the analyses outlined above it is clear that NtSyr1 associates with several putative interacting partner proteins and that several of these show differential interaction(s) when eluted with the Sp2 and Sp3 domains of the syntaxin (Fig. 1). A number of these proteins, especially the larger molecular weight fractions of roughly 50–100 kDa, seem to show a very high affinity for the Sp2 ligand and were recovered in significant quantities as well-defined bands only after eluting Sp2 affinity columns with the soluble Sp2 fragment (see Fig. 1). The nature of these proteins remains unknown and will be a major target for further exploration. Their characteristics as membrane proteins with high affinities for NtSyr1 could imply roles in cellular signal cascades – including ion channel functions for transport of K^+, Cl^- and Ca^{2+} – as well as in vesicle trafficking, cytoskeletal and homeostatic activities [21–29].

The differences in Sp2 and Sp3 elutions, and the similarities between KCl elutions from Sp2 and Sp3 columns, are not surprising since the salt, which affects global charge interactions, will be less specific in its action. Selectivity between Sp2 and Sp3 eluates, on the other hand, is likely to reflect competition for binding partners between protein fragments in solution and covalently bound to the column. The H3 domain (absent from the Sp3 fragment) contributes one of the four helices comprising the SNARE complex [6,30] as well as interacting with other proteins including ion channels [2]. It is also thought to interact with a regulatory complex formed of the HA, HB and HC domains in the absence of vesicle fusion [7,31]. The HA/HB/HC domains, in turn, may also interact with discrete partners, both regulatory proteins [7,32] and, in the absence of the H3 domain, also ion channels [33]. So, both the H3 and HA/HB/HC domains are potential targets for protein–protein interactions with NtSyr1 partners.

Of the lower molecular weight proteins, at least five bands are likely to include components of SNARE complexes on the basis of their sensitivity to proteolysis by *C. botulinum* neurotoxins (Figs. 2 and 4) and cross-reactivity with antibodies prepared against the *Arabidopsis* plasma membrane SNARE element AtSNAP33 (Fig. 3). The tobacco syntaxin, itself, is a target for proteolysis by BotN/C [11]. We found that distinct protein bands near 18, 22, 55 and 62 kDa were lost in the presence of BotN/A and/or BotN/C toxins while a new band appeared around 23 kDa following treatments with BotN/A and BotN/E toxins. One or more proteins near 55 kDa cross-reacted with both the αAtSNAP33 and αSp2 antibodies, both in the total microsomal fraction and in the Sp2 affinity eluate, and treatments of microsomal membranes with BotN/A and BotN/E toxins led to the appearance of a prominent αAtSNAP33-reactive bands around 23 kDa. AtSNAP33, itself, proved sensitive to proteolysis specifically by these neurotoxins and cleavage of AtSNAP33 (not shown) and the 55 kDa band (Fig. 4A) required the presence of MgATP, consistent with the activity of NSF ATPase in dissociating complexed SNARE proteins that are otherwise protected from proteolytic cleavage by BotN/*x* toxins [18]. We suspect, therefore, that these bands comprise or include one or more SNAP25 homologues of tobacco. Which of the bands may correspond to or include SNAP25 homologues of tobacco and which is likely to be the in vivo partner of NtSyr1 remains to be determined. The *Arabidopsis* genome encodes three distinct SNAP25 homologues, including AtSNAP33 [8], and it is plausible that the tobacco genome encodes at least as many.

In summary, we find that the cytosolic domain of NtSyr1 interacts strongly with a number of binding partners with

molecular weights between 18 and roughly 100 kDa. Of these, at least four protein fractions showed susceptibility to proteolysis by *C. botulinum* neurotoxins in patterns characteristic of t-SNAREs such as SNAP25, and one fraction cross-reacted with antibodies raised against the *Arabidopsis* SNAP25 homologue AtSNAP33. These observations imply significant structural similarities between mammalian, *Arabidopsis* and tobacco SNAREs. They also demonstrate the utility of the affinity chromatographic method for further purification and analysis of SNARE proteins and their interactors in plants.

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