

CHAPTER 3

INVESTIGATIONS INTO THE CONSERVED CORE MACHINERY OF REGULATED EXOCYTOSIS IN THE SALIVARY GLANDS OF *O. savignyi*

3.1. INTRODUCTION

In essentially all cells a process known as constitutive exocytosis occurs, where secretory vesicles fuse to the plasma membrane in order to insert new cell membrane and membrane components. In many cells a second pathway, termed regulated exocytosis, also exists where fusion occurs only in response to a physiological signal (Burgoyne and Morgan 2003). To date, the process of regulated exocytosis has been most extensively studied in cells with a crucial physiological or pathophysiological interest. The wide variety of secretory granule-containing cells in mammalian species are shown in Table 3.1.

Table 3.1. Cells with secretory granules (Burgoyne and Morgan 2003)

Class of cell	Cell Type	Granule
Neuron	Various	Dense-core vesicles
Endocrine neurons	Hypothalamic Posterior pituitary Median eminence	Large dense core-vesicles Large dense core-vesicles Large dense core-vesicles
Neuroendocrine/endocrine cells	Adrenal chromaffin Anterior pituitary Atrial myocytes Carotid body glomus cells Gut enteroendocrine cells Gastric enterochromaffin-like cells Gastric G cells Intestinal I cells (cholecystokinin) Kidney juxtaglomerulal cells (rennin) Pancreatic A cells (glucagon) Pancreatic B cells (Insulin) Pancreatic D cells (Somatostatin) Pancreatic F cells (Pancreatic polypeptide) Parathyroid cells (Parathyroid hormone) Pineal glands Posterior pituitary Thyroid C cells (calcitonin)	Chromaffin granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules Secretory granules

Exocrine	Airway goblet cells Alveolar type II epithelial cells Gastric chief cells Intestinal goblet cells Lacrimal Mammary epithelial cells Pancreatic acinar cells Parotid Submaxillary	Mucin granules Lamellar body Secretory granules Mucin granules Zymogen granules Casein vesicles Zymogen granules Zymogen granules Zymogen granules
Hemopoietic	Basophils Eosinophils Macrophages Mast cells Neutrophils Platelets T-cells	Lysosome-related granule Lysosome-related granule Multiple granules Lysosome-related granule Multiple granules Lysosome, dense and α -granules Lysosome-related granule
Other	Endothelial cells Melanocytes Sperm Egg All cell types	Weibel-Palade body Melanosomes Acrosome Cortical granules Lysosomes

3.1.1. Conserved core machinery for regulated exocytosis

The idea of stimulation-secretion coupling during regulated exocytosis, with calcium as the key trigger, was generally accepted by the 1970's (Burgoyne and Morgan 2003). The downstream mechanisms leading to membrane fusion was, however, unknown. The first enhancement to our understanding of membrane fusion came from the work of Novick and Schekman who isolated the first secretory (sec) mutant *sec1* from mutagenized yeast cells, after screening for temperature-sensitive secretion mutants (Novick and Schekman 1979). The second breakthrough came from the work of Rothman who identified NSF, which was purified from isolated Golgi complexes and cytosol based on its ability to reconstitute intra-Golgi transport after blockage by N-ethylmaleimide (Rothman 1994). Analysis of the NSF gene indicated that the coding sequence of NSF is 48% identical to that of the yeast *SEC18* gene. Later, it was shown that *sec18* could substitute for NSF in mammalian *in vitro* assays of endosome fusion, intra-Golgi transport and granule exocytosis. This suggests that NSF and *sec18* contribute similar functions to an evolutionary ancient molecular mechanism of membrane fusion in all cells (Burgoyne and Morgan 2003).

This hypothesis was supported when protein-protein interaction studies revealed that NSF and its co-factor, α -soluble NSF attachment protein (α -SNAP; Sec17 in yeast), interact with membranes via a SNAP receptor (SNARE) complex comprising VAMP/synaptobrevin (Snc1/2 in yeast), syntaxin (Sso1/2 in yeast) and SNAP-25 (Sec9 in yeast). Realization that two additional protein families, namely the Rabs and the Sec1 homologs, were also involved in multiple vesicular transport processes led to the concept of a universal mechanism of membrane fusion (Burgoyne and Morgan 2003). The key proteins involved in regulated exocytosis are listed in Table 3.2.

Table 3.2. Key proteins that function in exocytosis in neurons and in secretory granule exocytosis (Burgoyne and Morgan 2003)

Protein class	Neuronal Protein	Endocrine	Homologues in non-neuronal cells
SNAREs	Syntaxin SNAP-25 VAMP (Synaptobrevin)	Yes Yes Yes	Syntaxin isoforms SNAP-23 Yes
SNARE regulators	α -SNAP NSF Munc18 / nSec1 Munc 13	Yes Yes Yes Yes	Yes Yes Isoforms ?
Rabs and effectors	Rab3 Rabphilin Rim Noc2	Yes Yes Yes Yes	Other Rabs Yes ? Endocrine cells
Calcium binding proteins	Synaptotagmin Calmodulin CAPS	Yes Yes Yes	Yes Yes ?
Others	Csp Synapsins	Yes No	Yes Neuron specific

a. The SNAREs

SNAREs are membrane proteins that are localized to various intracellular organelles and are identified by a characteristic heptad repeat sequence known as the SNARE motif. Three proteins namely syntaxin, VAMP/synaptobrevin (vesicle associated membrane protein) and SNAP-25 form a SNARE complex, first described in synapses by Sollner *et al.* (Sollner *et al.* 1993). Since VAMP is localized on the vesicle membrane and syntaxin and SNAP-25 localize to the plasma membrane, SNAREs were originally classified as vesicle (v-) or target (t-)

SNAREs. More recently a rival classification system has been proposed based on whether a conserved glutamine (Q) or arginine (R) is present in the SNARE motif in the so-called zero layer (Figure 3.1.). In the SNARE complex, syntaxin contributes one Q-containing helix; SNAP-25 contributes two Q-containing helices and VAMP one R-containing helix. Characterized SNARE complexes always contain three Q-helices and one R helix. This code of 3Q:1R helices has been shown to be an essential combination which, in a lock-and-key fashion, allows specific SNARE pairing and consequent membrane fusion (Katz and Brennwald 2000). The Q and R residues are not essential for membrane fusion, but mutation impairs disassembly of the SNARE complex by NSF and α -SNAP (Graham *et al.* 2001).

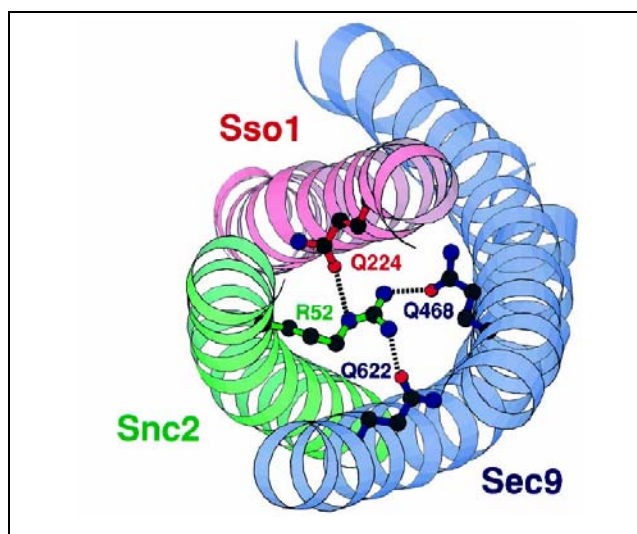


Figure 3.1. Model of the ionic layer of the yeast post-Golgi SNARE complex (Katz and Brennwald 2000). The yeast post-Golgi SNARE complex is a parallel four-helix bundle with most of the layers composed entirely of hydrophobic residues. Roughly halfway along the axis of the helical bundle is a unique ionic layer (also known as zero layer) consisting of an arginine and three glutamines. Within this layer, the arginine is contributed by the v-SNARE Snc2 (synaptobrevin) and the glutamines are contributed by the t-SNAREs Sec9 (SNAP-25) and Sso1 (Syntaxin). The glutamine and arginine residues form an extensive network of hydrogen bonds with each other. The flanking hydrophobic layers stabilize the ionic layer by protecting the polar interactions from exposure to the surrounding environment.

By using either mutational studies, clostridial neurotoxins, SNARE-directed antisera or peptides it could be shown that SNAREs are essential for regulated granule exocytosis in various cell types such as adrenal chromaffin cells, PC12 cells, pancreatic B-cells, pancreatic acinar cells, AtT-20 cells, eosinophils, platelets, peptidergic hypothalamic neurons, gastric enterochromaffin-like cells and sperm (Burgoyne and Morgan 2003). Based on various observations, it is believed that SNAREs are involved in regulated exocytosis by driving

membrane fusion. The accepted model comprises of four steps. First, syntaxin is unavailable for SNARE complex formation due to its association with munc18. Secondly, dissociation of munc18 renders syntaxin in the open conformation. Thirdly, the SNAREs interact and assemble into the four-helical bundle. Finally, as the complex is fully assembled, the lipid bilayers of the plasma membrane and vesicle are brought close enough for bilayer fusion to occur.

Syntaxin

All eukaryotes examined to date contain syntaxin-like sequences. In mammals, the syntaxin family consists of 15 genes and in yeast of 7 genes (Teng *et al.* 2001). Alternative splicing generates additional diversity within the family. All mammalian syntaxins (excluding syntaxin 11) are transmembrane proteins (~35 kDa) anchored by their C-terminal tail; therefore the amino-terminus and polypeptide face the cytoplasm. The SNARE-domain of approximately 60-residues is conserved in all syntaxins and mediates interactions with SNARE domains of other target membrane SNARE proteins (Teng *et al.* 2001). These include other syntaxins, SNAP-25 and VAMP. The amino terminus of some syntaxins serves as an auto-inhibitory domain by folding back onto the membrane proximal SNARE domain. In this case, the formation of a fusion complex is inhibited and the molecule is said to be in a 'closed' configuration (Figure 3.2). The chaperone protein n-Sec1 / munc18 binds to this closed conformation of syntaxin and dissociates upon a structural change induced by Rab GTPases to yield an 'open' configuration (Teng *et al.* 2001; Burgoyne and Morgan 2003).

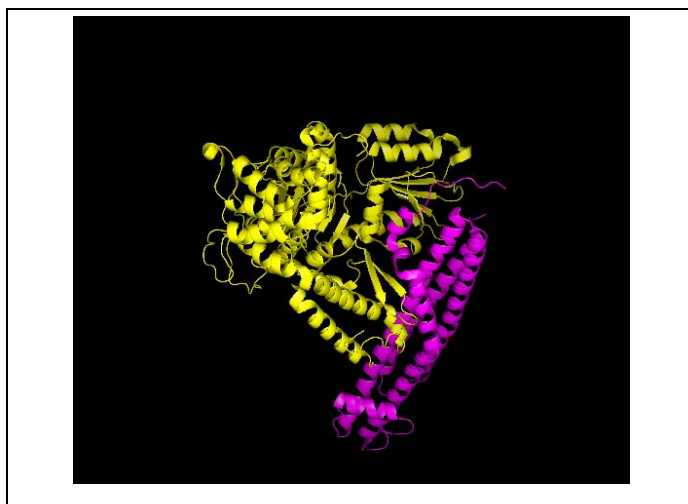


Figure 3.2. Crystal structure of the neuronal Sec1/syntaxin 1a complex (Misura *et al.* 2000). *The four helical bundles of syntaxin (purple) and nSec1 (yellow) bound to the N-terminal of syntaxin are shown.*

Syntaxins are localized to various subcellular locations where they function in anterograde, endocytotic/retrograde flow of traffic and known membrane transport steps (Table 3.3). They have also been shown to interact with a range of proteins other than their SNARE partners. These include vesicle coat proteins (Teng *et al.* 2001), Rab GTPases and effectors (Takai *et al.* 2001; Torii *et al.* 2002), tethering factors (Takai *et al.* 2001), channels such as the sodium channel, CFTR chloride channel (Kleizen *et al.* 2000) and voltage-sensitive calcium channels (Atlas 2001), as well as munc proteins (Betz *et al.* 1997; Kauppi *et al.* 2002).

Table 3.3. Cellular and functional information about mammalian syntaxins (Teng *et al.* 2001).

Isoform	Cellular Localization	Tissue Distribution	Known Function
Syntaxin 1 (A, B and C)	Presynaptic plasma membrane	Neuronal cells Secretory cells	Neuronal exocytosis Regulated exocytosis
Syntaxin 2 (A, B, C and D)	Plasma membrane	Ubiquitous	Exocytosis Morpho-regulator during development
Syntaxin 3 (A, B, C and D)	Plasma membrane	Ubiquitous	Exocytosis
Syntaxin 4	Plasma membrane	Ubiquitous	Glut4 translocation
Syntaxin 5 (Long and short form)	ER-Golgi boundary	Ubiquitous	ER-Golgi transport
Syntaxin 6	TGN	Ubiquitous	TGN-endosome transport Endosome-TGN transport Fusion of immature secretory granules
Syntaxin 7	Endosome	Ubiquitous	Late endosome fusion Late endosome-lysosome fusion
Syntaxin 8	Endosome	Ubiquitous	Late endosome fusion
Syntaxin 10	TGN	Ubiquitous	Unknown
Syntaxin 11	TGN / Late endosome	Ubiquitous	Unknown
Syntaxin 12 / 13	Endosome	Ubiquitous	Recycling of surface protein Early endosome fusion
Syntaxin 16 (A, B and C)	Golgi / TGN	Ubiquitous	Early endosome-TGN transport
Syntaxin 17	Smooth ER	Steroidogenic tissues	Trafficking to smooth ER
Syntaxin 18	ER	Unknown	ER-Golgi transport ER homotypic fusion

A recent study by Karim *et al.*, identified syntaxin isoforms 1 and 2 in the salivary gland of the lone star tick, *A. americanum* by means of Western blotting. Both syntaxin isoforms 1

and 2 proteins have a molecular mass of 35 kDa (similar to that in brain extracts) and are enriched in the membrane fraction obtained by centrifugation at 100,000 x g (Karim *et al.* 2002). Preliminary studies using partially purified plasma membrane indicated that tick syntaxins are localized on the plasma membrane since they co-localize with the Na⁺/K⁺-ATPase following sucrose density centrifugation (Karim *et al.* 2002).

Synaptobrevin / VAMP (Vesicle associated membrane protein)

The synaptobrevin / VAMP family members are small type II membrane proteins of about 120 amino acids (~18 kDa). They consist of a variable region of 25-35 amino acids located at the amino terminus, followed by either one extended or two short (helix 1 and 2) amphiphatic α -helical segments, which form coiled-coil structures and have a transmembrane domain located at their carboxyl terminus (Gerst 1999). Helix 1 is unusually hydrophobic and may interact with lipids during the process of membrane fusion while helix 2 is involved in SNARE-SNARE interactions (Grote *et al.* 1995). Various isoforms and spliced variants have been identified to date (Table 3.4).

Table 3.4. Cellular and functional information of synaptobrevins (Gerst 1999; Jahn 1999).

Protein	Synonym	Localization	Trafficking event
Synaptobrevin 1	VAMP1	Synaptic vesicles Clathrin-coated vesicles	Regulated exocytosis
Snc1 and Snc2	Yeast equivalent of VAMP1 / 2	Plasma membrane	Golgi to Plasma membrane
Synaptobrevin 2	VAMP2	Synaptic vesicles / Secretory granules Clathrin-coated vesicles	Regulated and Constitutive exocytosis
Cellubrevin	VAMP3	Ubiquitous Microvesicles Clathrin-coated vesicles	Regulated and Constitutive exocytosis
VAMP 4	Synaptobrevin 4	Unknown	Unknown
VAMP 5/6	Synaptobrevin 5/6	Myogenesis skeletal muscle and heart Plasma membrane Intravesicular structures	Constitutive exocytosis
Ti-VAMP	VAMP 7 / Synaptobrevin-like protein	Apical membrane Secretory granules Endosomes	Regulated and Constitutive exocytosis
Endobrevin	VAMP 8	Early Endosomes	Endocytosis?

The study of Karim *et al.* also identified VAMP2 in the salivary glands of *A. americanum* (Karim *et al.* 2002). The protein identified by Western blotting with an anti-VAMP2 antibody, co-migrated with the 18 kDa VAMP protein in rat brain. By purifying secretory granules and vesicles by means of a two-step sucrose gradient centrifugation, it could be shown that VAMP-2 localize to the granule fraction (chromogranin B positive) and not the plasma membrane fraction.

SNAP 25

SNAP-25 belongs to a family of evolutionary conserved proteins whose members are essential for membrane fusion. SNAP-25 is a hydrophilic protein of 206 amino acids. It associates with membranes through palmitoyl residues, which are thioester-linked to four closely spaced cysteine residues at the centre of the protein (Hodel 1998). The N- and C terminals are highly conserved and are predicted to form intermolecular coiled coils. Proteolysis by botulinum toxins A, E and C1 truncates SNAP-25 by cleaving a single peptide bond near the C-terminus. Syndet and SNAP-23 are ubiquitously expressed homologues of SNAP-25 with a sequence homology of ~60%. These homologues are thought to mediate constitutive exocytosis by interacting with other isoforms of the SNAREs syntaxin and VAMP at the plasma membrane (Figure 3.3) (Hodel 1998).

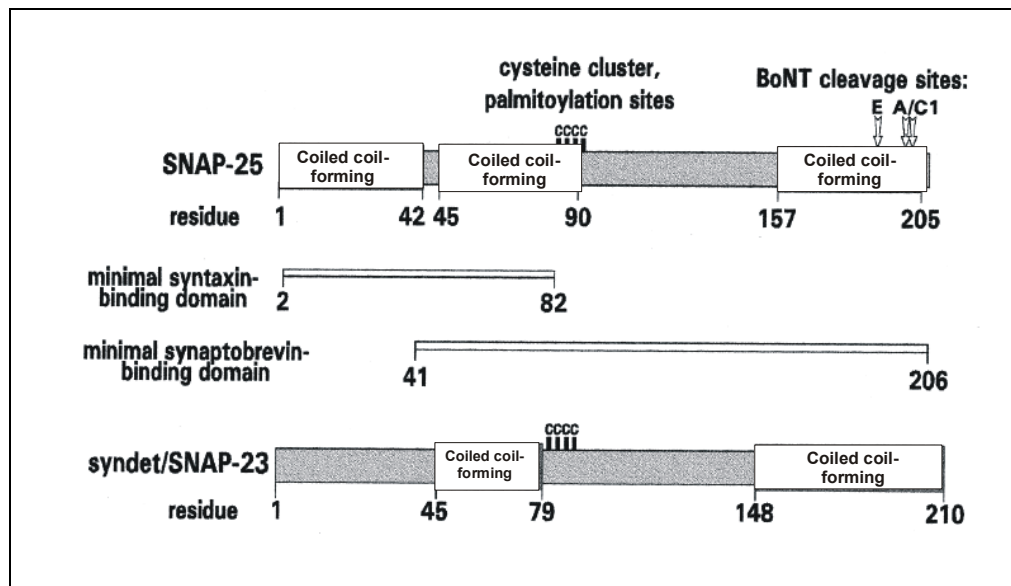


Figure 3.3. Protein structure of neuronal SNAP-25 and ubiquitously expressed homologues (Hodel 1998).

Although SNAP-25 is predominantly expressed in neuronal cells, it has been shown to be essential for the exocytosis of large dense core vesicles from neuroendocrine cells (Hodel 1998), PC12 cells (Banerjee *et al.* 1996b) and chromaffin cells (Tagaya *et al.* 1996). Apart from forming the SNARE complex, SNAP-25 has also been shown to bind and negatively modulate calcium channels (Hodel 1998). It also binds the calcium sensor synaptotagmin (Gerona *et al.* 2000). When part of the SNARE complex, SNAP-25 mediates subsequent binding of α -SNAP (Marz *et al.* 2003).

In a study by Castle *et al.* SNAP-23 has been implicated as a link between signaling and exocytosis in mast cells. Their findings indicated stimulus-induced relocation of SNAP-23 from foci in the plasma membrane to putative sites of membrane fusion both at the cell surface and intracellularly between granules (Castle *et al.* 2001).

Niemeyer and Schwarz have identified a new member of the family, SNAP-24 in *Drosophila*. Unlike SNAP-25, SNAP-24 is not concentrated in synaptic regions but can form complexes with both synaptic- and non-synaptic v-SNAREs. High levels of SNAP-24 were found in larval salivary glands, where they localized mainly to granule membranes. During a massive exocytotic event of these glands, SNAP-24 containing granules fused with one another and the apical membrane. This suggests that SNAP-24 is a mediator of secretion and granule-granule fusion in salivary glands (Niemeyer and Schwarz 2000).

b. NSF and SNAPs

NSF is a homo-oligomeric ATPase consisting of three 76 kDa subunits, which function together with SNAPs to disassemble SNARE complexes (Burgoyne and Morgan 2003). Owing to the extreme stability of the SNARE complex, disassembly requires ATP hydrolysis which is provided by NSF. Each identical subunit of NSF has a N-terminal domain required for binding to α -SNAP, followed by two ATPase domains (D1 and D2) with distinct, essential functions. The ability to hydrolyze ATP is unique to the D1 domain, whereas the function of the inactive D2 domain is to establish the oligomeric complex. Conformational changes in NSF upon ATP hydrolysis are transduced to α -SNAP to unwind the four helices of the SNARE complex. Apart from disassembling SNARE complexes, NSF has also been shown to function independently of SNAREs by binding different receptors (or their interacting proteins) and to regulate their insertion or removal from the plasma membrane. Such receptors include the AMPA receptor,

the β_2 -adrenergic receptor, β -arrestin and the GABA_A receptor-associated protein (Burgoyne and Morgan 2003).

Soluble NSF-attachment proteins (SNAPs) were identified in 1990 because of their ability to restore intracellular vesicular protein transport after salt extraction of Golgi-membranes (Stenbeck 1998). SNAPs are required to mediate membrane attachment of the cytosolic protein NSF. In mammals there are three isoforms, α -, β - and γ -SNAP. All are soluble and have molecular masses between 33-36 kDa. α and γ -SNAP are expressed ubiquitously while β -SNAP is restricted to the brain (Stenbeck 1998). In chromaffin cells, both α and β -SNAPs enhance exocytosis induced by micromolar calcium, but only α -SNAP does so at sub-micromolar calcium levels. Binding of α - and β -SNAPs to the SNARE complex induces a conformational change that renders it competent for binding and activating the ATPase activity of the D1 domain of NSF. The function of γ -SNAP remains unclear (Burgoyne and Morgan 2003).

To date, functional evidence supports a role for α -SNAP and/or NSF in granule exocytosis in chromaffin cells, PC12 cells, pancreatic β -cells, spermatozoa and platelets. Certain observations also suggest a chaperone function for NSF and α -SNAP, continually disassembling *cis* complexes (granule-granule) to release SNARE proteins to engage in *trans* (granule-plasma membrane) and hence further membrane fusion events (Burgoyne and Morgan 2003).

c. The Sec1 / Munc proteins

Genetic studies have established the importance of Sec-1 related genes in vesicle fusion in a wide variety of organisms such as yeast, plants, nematodes, flies and mice. Since the isolation of the first sec proteins from yeast (Sec1), three Sec1 orthologs have been isolated in mammals. These include the neuronal/endocrine munc18-1/nSec1 proteins, the ubiquitous munc18-2 and the munc18-3 isoforms. Sec1 family members from numerous organisms have been shown to interact with syntaxin homologs. In yeast, the syntaxins Sso1 and Sso2 act as suppressors of sec1-1 mutants while in neuronal tissue it has been shown that Sec1/munc18 bind syntaxin with nanomolar affinity *in vitro* to form a complex that precludes syntaxin-binding to other SNAREs. These observations suggest that Sec1 proteins act as syntaxin chaperones, preventing SNARE complex assembly until signaled to release syntaxin (Burgoyne and Morgan 2003).

Sec1 proteins have been implicated in granule exocytosis in pancreatic cells, platelets, pancreatic acinar cells, PC12 cells and chromaffin cells (Burgoyne and Morgan 2003). In the tick, *A. americanum*, it was shown that an anti-human nSec1 polyclonal antibody reacts with a 68 kDa protein in various salivary gland fractions. Several low molecular bands also cross-reacted both in the salivary gland fractions and the rat brain tissue, used as positive control (Karim *et al.* 2002).

d. Rabs and Rab-effectors

Rab GTPases (Ras-related in brain) belongs to the Ras family of small GTPases and form the largest branch of the small G protein superfamily (Stenmark and Olkkonen 2001). They exist in all eukaryotic cells, and to date more than 60 Rab proteins and isoforms have been identified in mammalian cells (Pfeffer 2001; Takai *et al.* 2001). At first it was thought that Rab proteins regulate intracellular vesicle trafficking, but it is now emerging that Rab proteins are key to the recruitment of membrane-tethering and docking factors that facilitate membrane traffic. In some cases, Rabs are important for the formation of transport vesicles and the recruitment of motor proteins onto vesicles to allow vesicle motility. But how do Rabs facilitate all these different functions? The answer lies in the various scaffolds built by the various Rabs (Pfeffer 2001).

Within cells, Rabs are reversibly localized between the cytosolic face of distinct intracellular membranes and the cytosol. This reversible location depends on the post-translational modification of a cysteine motif (CXXX, CC, CXC, CCXX or CCXXX where X is any amino acid) at the C-terminal, with one or more highly hydrophobic geranylgeranyl groups. This modification entails the recognition of the newly synthesized Rab by a Rab escort protein (REP), which presents it to the geranylgeranyl transferase. REP also functions as a chaperone that keeps the hydrophobic geranylated Rab soluble and delivers it to the appropriate membrane. The REP-Rab GTPase is in the GDP-form, but upon membrane delivery GDP/GTP exchange is catalyzed by a GDP/GTP exchange factor (GEF) that causes the release of the REP.

Rab proteins then cycle between the GDP-bound inactive and GTP-bound active forms and between the cytosol and membrane (Figure 3.4). At steady state, 10-50% of a given Rab protein is detected in the cytosol (Takai *et al.* 2001). Prenylated Rabs are bound to the GDP-GTP dissociation inhibitor (GDI) in the cytosol. This complex has the capacity to deliver the

Rabs to the correct compartment in their GDP-bound forms. A proteinaceous factor named GDI-displacement factor (GDF) releases a Rab from the GDI, permitting the Rab to insert into the adjacent membrane. Activation by a GDP-GTP exchange factor (GEF) follows and conversion to the GTP-bound form enables effector binding (Pfeffer 2001).

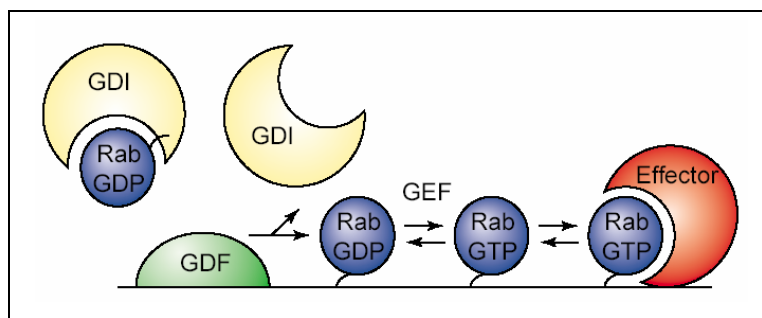


Figure 3.4. A model for Rab recruitment (Pfeffer 2001). Prenylated Rabs are bound to GDP-GTP dissociation factor (GDI) in the cytosol. This complex has the ability to deliver Rabs to the correct compartment in their GDP-bound forms. A GDI-displacement factor (GDF) releases a Rab from the GDI, permitting Rab to insert into the adjacent membrane. Activation by a GDP-GTP exchange factor (GEF) follows; conversion to the GTP-bound form enables effector binding.

Organelles have always been classified by the marker molecules present, for example in the Golgi stack where each cisterna houses a separate set of enzymes. The early Golgi contains mannosidase I, medial Golgi contains N-acetylglucosamine transferase I, and the late Golgi galactosyl transferase, and so on. For endocytic compartments, the classification was less clear until the realization that different compartments in the exocytic and endocytic pathways contain distinct Rab GTPases on their surfaces (Pfeffer 2001). Furthermore, Rabs are now known to collect integral and peripheral membrane proteins into a specific domain (or scaffold) on an organelle. One such an example is Rab1, which enables the Golgi to recognize incoming cargo by the GM130-GRASP65 scaffold. Rab1 has been shown to interact with a complex of GM130-GRASP65, thereby facilitating the delivery of transport vesicles to the Golgi (Pfeffer 2001). Rab1 also provides a link between vesicles leaving the ER and their destination (the Golgi) by binding to p115, a protein that interacts with the Golgi scaffold. The localization and function of some other Rabs are summarized in Table 3.5.

Numerous genetic and biochemical studies indicated that the key to the function of Rab proteins is the recruitment of effector molecules, which bind exclusively to Rabs in their GTP-form. Crystallographic data indicated that Rab proteins adopt two different conformations when in the GDP- and GTP-bound states and that binding between Rabs and their effectors

is via the switch domains. These domains are described as the two major nucleotide-induced conformation-changing domains and are located in loops 2 and 5 on the surface of the Rab protein. Rab effectors are a very heterogeneous group of proteins: some are coiled-coil proteins involved in membrane tethering or docking, while others are enzymes or cytoskeleton-associated proteins (Stenmark and Olkkonen 2001). The effectors recognized by some selected Rabs are listed in Table 3.5.

Rab proteins also interact with SNAREs. During *in vivo* studies it was shown by Grote and Novick that these interactions are non-selective in the absence of effector proteins (Grote and Novick 1999). Recent studies also indicated the important role of tethering/docking factors in this recognition process. Tethering factors involved in vesicle targeting/docking include Uso1, TRAPP, p115, exocyst and EEA1, and these all bind membranes before the formation of SNARE complexes (Takai *et al.* 2001). EEA1, which is involved in the homotypic endosome fusion process, has recently been found to be an effector of Rab5. It is likely that the events that precede stable SNARE-dependent docking of membranes are the result of a network of interactions between many proteins including tethering and Rab proteins. Direct interactions between Rabs and Sec4 as well as Rabs and the exocyst have also been described. Therefore Rabs are likely candidates for orchestrating vesicle targeting through tethering proteins (Takai *et al.* 2001).

To date, no tethering proteins have been identified in tick tissues. Karim *et al.* did however identify a 25 kDa putative Rab3 in the salivary glands of *A. americanum* using a polyclonal antibody to human Rab3a. The molecular mass of the identified tick protein was similar to that observed in rat brain. Various fractions, such as the pellet and supernatant obtained after a 2000 x g centrifugation fractionation and the pellet obtained after 150,000 x g centrifugation, tested positive. The supernatant obtained after 150,000 x g, the plasma membrane fraction obtained from sucrose gradient membrane fractionation and saliva, tested negative (Karim *et al.* 2002).

Table 3.5. Localization, function and effectors of selected Rab GTPases (Watson 1999; Pfeffer 2001; Stenmark and Olkkonen 2001; Takai *et al.* 2001). *Subcellular compartment abbreviations: ER (endoplasmic reticulum), SV (synaptic vesicles), EE (early endosome), CCV (clathrin coated vesicles), PM (plasma membrane), LE (late endosome). TGN (trans Golgi network) and RE (recycling endosome). Effector abbreviations: RIM (rab interacting protein), EEA (early endosome associated protein).*

Name	Yeast Homologue	Localization	Expression	Function	Effector
Rab1a	Ypt1p	ER / <i>cis</i> -Golgi	Ubiquitous	ER-Golgi transport	
Rab2a		ER / <i>cis</i> -Golgi	Ubiquitous	Golgi-ER retrograde transport	
Rab3a Rab3		SV Granules	Neurons Ubiquitous	Regulated exocytosis	Rabphilin-3A RIM
Rab4a		EE	Ubiquitous	Endocytic recycling	
Rab5a	Ypt51p	EE, CCV, PM	Ubiquitous	Budding, motility and fusion in exocytosis	Rabaptin-5 Rabenosyn-5 EEA1
Rab6a	Ypt6p	Golgi	Ubiquitous	Retrograde Golgi traffic	Rabkinesin-6
Rab7	Ypt7p	LE	Ubiquitous	Late endocytic traffic	
Rab8a	Sec4p	TGN, PM	Ubiquitous	TGN-PM traffic	Pab8ip (Ser/ Thr kinase)
Rab9a		LE	Ubiquitous	LE-TGN traffic	p40
Rab11a	Ypt31p	RE, TGN	Ubiquitous	Endocytic recycling via RE and TGN	Rab11BP/Rabphilin-11
Rab27a		Melanosomes Granules	Melanocytes Platelets Lymphocytes	Movement of lytic granules and melanosomes towards PM	

e. Calcium binding proteins

The near universal role of calcium as the trigger for granule exocytosis predicts the existence of conserved protein(s) capable of activating the fusion machinery upon binding calcium (Burgoyne and Morgan 2003). Several different candidate proteins have been suggested to play such a role. Since synaptotagmins have attracted most of the attention, they will be discussed in more detail.

Synaptotagmins belong to the large family of C2 domain proteins since it contains two identical repeats that share homology with the protein kinase C (PKC) C2 domain. To date they have been detected in numerous eukaryotes but have not been found in yeast, suggesting that they are not part of the basic membrane trafficking machinery (Marqueze *et al.* 2000).

Synaptotagmin I (sytI) is abundantly expressed in neuronal and endocrine cells where they localize to the synaptic vesicles and secretory granules, respectively. Synaptotagmin I interacts with various binding partners (Figure 3.5) including the key components of the exocytosis-endocytosis machinery: syntaxin (Chapman *et al.* 1995), SNAP25, β -SNAP as well as various channels, lipids, neuexins (Perin 1996), the clathrin adaptor protein AP-2 (Haucke and De Camilli 1999) and calmodulin (Perin 1996). These interactions are regulated via phosphorylation by casein kinase II, calmodulin kinase II and PKC. Although sytI is most abundant in neuronal tissue, it has been detected in other tissues such as chromaffin cells where it localizes to the large dense core vesicles (LDCVs). By means of sytI deficient cells, it could be shown that sytI is required for rapid, highly calcium sensitive LDCV exocytosis, and that it regulates the equilibrium between a slowly releasable and a readily releasable state of the fusion machinery (Voets *et al.* 2001).

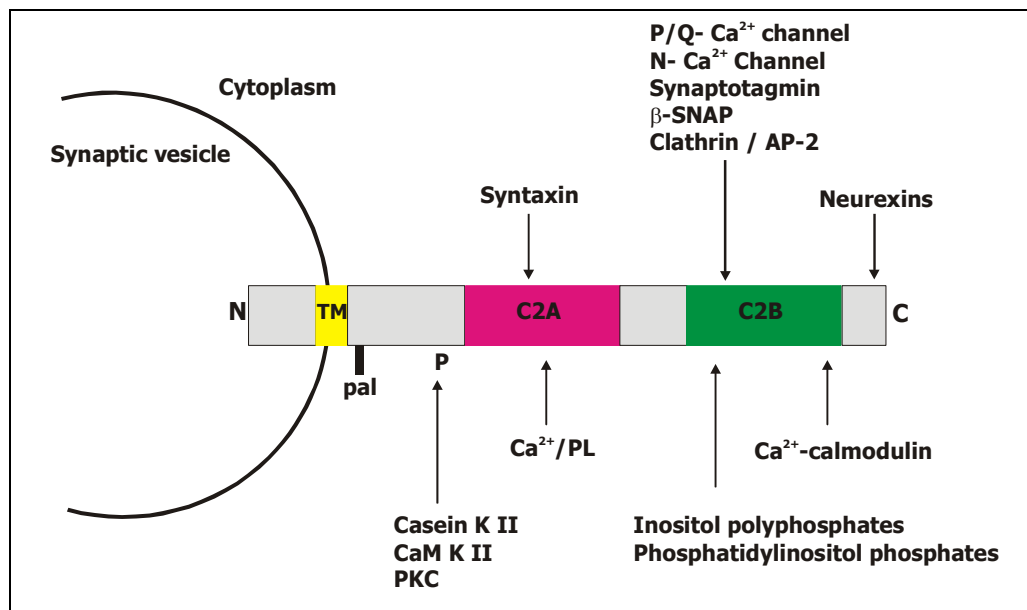


Figure 3.5. Diagram of the domain structure of synaptotagmin I (Marqueze *et al.* 2000). The interaction sites with the major binding partners are indicated. Abbreviations corresponds to: (C) carboxy-terminus; (CaM Kinase II) Ca²⁺/calmodulin kinase II; (Casein K II) casein kinase II; (N) amino-terminus; (P) phosphorylation sites; (pal) palmitoylation site; (PL) phospholipids; (PKC) protein kinase C and (TM) transmembrane domain.

Synaptotagmin II (sytII) shares the highest homology with sytI and is assumed to also function similarly. Hetero-oligomers have been described and proposed to modulate the properties of the synaptotagmins in neuro-secretion. Properties of the other synaptotagmin isoforms are listed in Table 3.6.

Table 3.6. Properties of various synaptotagmin isoforms (Marqueze *et al.* 2000; Sudhof 2002).

Subcellular compartment abbreviations: SLMVs: synaptic like microvesicles, LDCVs: Large dense core vesicles.

Isoform	cDNA identification	Protein expression, properties and function
I	Peptides of the protein were used to design oligonucleotides and screening of a cDNA library	<ul style="list-style-type: none"> -Synaptic vesicles from rat brain - SLMVs from adrenal medulla - LDCVs from PC12 cells, posterior pituitary - Secretory granules of insulin secreting cells - Parotid secretory granules - Ca²⁺ sensor for fast exocytosis - N-glycosylated N terminus
II	Screening of a genomic library using sytI probes	<ul style="list-style-type: none"> - Growth cones - Synaptic vesicles - Secretory granules of insulin secreting cells - Ca²⁺ sensor for fast exocytosis - N-glycosylated N terminus
III	RT-PCR with sytI primers	<ul style="list-style-type: none"> - Mouse neuromuscular junction - Secretory vesicles of pancreatic B-cells - Ca²⁺ sensor for exocytosis - Disulfide bonds at N-terminus - Determine secretory granule size (Grimberg <i>et al.</i> 2003)
IV	<ul style="list-style-type: none"> - Differential screening of an induced PC12 cDNA library - PCR with C2 domain degenerative primers 	<ul style="list-style-type: none"> - Rat brain membranes (62 kDa) - Drosophila heads membranes (55 kDa) - SLMVs and LDCVs of PC12 cells (46 kDa) - Unknown function - Asp → Ser substitution in C2A-domain
V	Screening of a cDNA library with sytI probe (Hudson and Birnbaum 1995)	<ul style="list-style-type: none"> - Kidney, Adipose tissue, lung, Heart - Ca²⁺ sensor for exocytosis - Disulfide bonds at N-terminus
VI	Screening of a libraries using syt probes	<ul style="list-style-type: none"> - ~50 KDa protein - Not in synaptic vesicles - Intestine, Kidney, Pancreas, Testis - Ca²⁺ sensor for exocytosis - Disulfide bonds at N-terminus - No consensus Ca²⁺ binding sites
VII	Screening of a libraries using syt probes	<ul style="list-style-type: none"> - Brain (several proteins), Heart, Lung, Spleen - Ca²⁺ sensor for exocytosis ->12 splice isoforms

VIII	Screening of a libraries using syt probes	- Renal cortex and medulla (52 kDa), Heart, Cerebral cortex - No consensus Ca ²⁺ binding sites
IX (sytV)	-Screening of rat adipose tissue cDNA library with sytI probe -E17 rat forebrain cDNA library	- Brain, PC12 cells (50 kDa), Adipose tissue, Heart, Kidney - Unknown function
X	Differential display PCR	- Dentate granule cells of cortex after kainic acid-induced seizures - Ca ²⁺ sensor for exocytosis - Disulfide bonds at N-terminus
XI	PCR with primers designed in a human syt EST sequence	- Brain - Unknown function - Asp→ Ser substitution in C2A-domain
<i>srg1</i>	Screening of cDNA library for thyroid hormone up-regulated genes	- Brain

The various isoforms differ mostly regarding their calcium-dependent phospholipid binding capability, binding to other synaptotagmins, calmodulin and AP-2 binding as well as the EC₅₀ of the C2A domain for calcium. The ability to interact with both membranes and the fusion machinery in response to the spectrum of calcium levels that drive exocytosis, suggests that synaptotagmins may be ubiquitous exocytotic calcium-sensors (Marqueze *et al.* 2000; Burgoyne and Morgan 2003).

It is possible that various classes of calcium-sensors determine the distinct calcium dependency of granule exocytosis. PKC, rabphilin 3A, Doc2, RIM and munc13, all proteins involved in exocytosis, share the C2 domains of synaptotagmin. Although rabphilin has originally been isolated as a Rab3 binding protein, recent studies indicate a function independently of Rab3 in various systems during exocytosis. Doc2 (double C2) overexpression in PC12 cells indicated its involvement in granule exocytosis by interacting with both munc18/nSec1 and another C2 domain containing protein, munc13. Overexpression of munc-13 in chromaffin cells increases the magnitude of both the exocytotic burst and the subsequent slower phase of release, suggesting a role in priming. The final C2 domain-containing protein implicated in exocytosis is RIM (Rab Interacting Molecule). Studies in PC12 cells, β -cells, β -cell lines and chromaffin cells indicated a function for RIM in exocytosis that is unrelated to Rab3 (Burgoyne and Morgan 2003).

Two proteins, calmodulin and p145/CAPS, that lack C2 domains have also been implicated in granule exocytosis. Calmodulin binds calcium via its four EF-hand domains and was one of the first proteins suggested to be involved in the late calcium-activated triggering of exocytosis. Its mechanism of action is unclear as it binds to several proteins implicated in exocytosis, including synaptotagmin I, Rab3, VAMP and the SNARE complex. Recently, p145/CAPS has emerged as a granule exocytosis-specific calcium sensor. Originally identified as a cytosolic factor that reconstituted secretion from GH3 and PC12 cells, CAPS has since been shown to regulate dense-core vesicle exocytosis in nerve terminals, chromaffin cells and pituitary melanotrophs. CAPS is known to act in the late calcium-activated triggering stage, but its mechanism of action is unknown. In particular, its calcium affinity ($K_d=270 \mu\text{M}$) appears too low to explain its effect on granule exocytosis (Burgoyne and Morgan 2003).

To date, the only calcium-sensor detected in ticks is synaptotagmin using western blotting and an rabbit-anti-rat synaptotagmin polyclonal antibody (Karim *et al.* 2002). The molecular mass of 65 kDa is similar to synaptotagmins I and II found in brain tissue.

3.2. HYPOTHESIS

- Salivary glands of the soft tick *Ornithodoros savignyi* contain homologues of the conserved core machinery involved in regulated exocytosis, i.e. syntaxin, SNAP-25 and VAMP.

3.3. AIMS

- Identification of the conserved core machinery (syntaxin, SNAP-25 and VAMP) and the Rab3 GTPase by means of Western Blotting using polyclonal antibodies against the rat brain isoforms.
- Localization of the SNARE proteins using subcellular fractionation by ultra-centrifugation and confocal microscopy.
- Isolation of the cDNA transcripts encoding the SNARE protein syntaxin and the calcium-binding protein synaptotagmin from argasid ticks by means of degenerative primers and 3'-RACE.

3.4. MATERIALS

New Zealand white rabbits were obtained from the NICD (National Institute for Communicable Diseases), Johannesburg, South Africa. The rabbits resided at the animal unit of the Medical Research Council (MRC), Pretoria, South Africa. All animal studies were also conducted at the MRC with the help of Mr. K. Venter. Recombinant synaptotagmin was a kind gift from Prof. R.H. Scheller, Stanford University Medical Center, USA. Diethyl pyrocarbonate (DEPC) and dithiothreitol (DTT) were obtained from Sigma Chemical Co. Tris(hydroxymethyl)aminomethane, NaCl, ethylene diamine tetra-acetic acid (EDTA), methanol, acetic acid, glycine, ammonium persulphate, N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Merck, Darmstadt, Germany. Acrylamide, bisacrylamide, sodium dodecyl sulphate (SDS) were from BDH Laboratory Supplies LTD., England. Chemiluminescent molecular weight marker proteins, Super Signal[®] chemiluminescent substrate and Protein Assay kit were from Pierce, USA (Separations). TaKaRa Ex Taq (5 U/ μ l) and Taq (5 U/ μ l) were from Takara Bio Inc., Japan (Separations). X-ray film, photographic developer and fixer were from Konica. The RNeasy total RNA isolation kit and the Super Smart cDNA synthesis kit were obtained from Qiagen (Southern Cross Biotechnology). SNARE antibodies were a gift from Prof. J. R. Sauer, Oklahoma State University, USA. All primers were synthesized by Inqaba Biotech, Pretoria, South Africa. Isopropyl β -D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal), RNase, High Pure plasmid isolation kit, RNase-inhibitor, PCR nucleotide mix (10 mM deoxynucleotides solution), DIG (Digoxigenin) labeled dUTP, blocking solution, anti-Digoxigenin alkaline phosphatase and CDP-Star[™] were from Roche Diagnostics. Yeast extract and tryptone were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England). SuperScript II Rnase H⁻ reverse transcriptase was from Invitrogen Life Technologies.

3.5. METHODS

3.5.1. SALIVARY GLAND FRACTIONATION

Salivary glands (10 pairs) were homogenized in 1 ml extraction buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 2.5 mM EDTA, 1 μ g/ml leupeptin, 20 μ g/ml aprotinin, 0.5 mM phenylmethylsulfonylfluoride) and fractionated by ultra centrifugation (Sorvall[®], Ultra Pro[™] 80, Du Pont) for 60 minutes at 4^o C. Fractions were collected after the 2000 x g and 100 000 x g centrifugation steps and protein concentrations determined. Protein fractions (100 μ g) were separated by SDS-PAGE and subjected to Western blotting.

3.5.2. PROTEIN GEL ELECTROPHORESIS

SDS-PAGE was performed using a 5% stacking gel (0.625 M Tris-HCl, 0.5% SDS, pH 6.8) and 12% separating gel (1.88 M Tris-HCl, 0.5% SDS, pH 8.8). The acrylamide gels and the electrophoresis buffer were prepared from an acrylamide stock (30% acrylamide, 0.8% N',N'-methylene bisacrylamide) and electrophoresis buffer stock (0.02M Tris-HCl, 0.06% SDS, 0.1 M glycine, pH 8.3). The gel solutions were polymerized with the addition of 30 µl of 10% ammonium persulphate and 5 µl TEMED. Samples were diluted 1:1 in reducing sample buffer (0.06 M Tris-HCl, 2% SDS, 0.1% glycerol, 0.05% β-mercaptoethanol, 0.025% bromophenol blue) and boiled at 95°C for 5 minutes. Pre-stained molecular mass markers (Pierce, USA) were dissolved in 10 µl water. Electrophoresis was carried out in a Biometra electrophoresis system (Biometra GmbH, Germany) with an initial voltage of 60 V for 45 minutes and thereafter 100 V. When chemiluminescent Blue Ranger® Prestained Peroxidase-labeled protein molecular weight markers (Pierce, USA) were used, SDS was omitted from the gels and the marker sample was not boiled.

3.5.3. WESTERN BLOTTING

Proteins were blotted onto PVDF (polyvinylidene-difluoride, Merck) membranes using a Trans-Blot semi-dry transfer cell (Bio-Rad) and 10 mM CAPS (3-(cyclohexylamino)-1-propane sulphonic acid) at 20 V for 45 minutes. Membranes were blocked with 1% skim milk powder in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4-7.6) overnight at 4°C. Blots were then incubated with the primary antibodies at a dilution of 1:1000 in blocking buffer. The antigen-antibody complexes were visualized with alkaline peroxidase conjugated anti-rabbit or anti-mouse IgG (whole molecule) at a dilution of 1: 10 000 and detected with Super Signal® chemiluminescent substrate (Pierce, USA).

3.5.4. IMMUNO-FLUORESCENT LOCALIZATION USING CONFOCAL MICROSCOPY

Glands of unfed female ticks were dissected in PBS and immediately fixed in 1% formaldehyde and 0.1% glutaraldehyde for 3 hours. These fixed glands were permeabilized in a graded series of methanol solutions (1-100%) before rehydration in PBS. Glands were subsequently incubated overnight in 100 mM NaBH₄ at 4°C with the Eppendorf caps open to minimize auto-fluorescence. Glands were washed 3 times with TBSN buffer (10 mM Tris-HCl, pH 7.4, 155 mM NaCl, 0.1% Triton X100) for 90 minutes with constant rotation before blocking with 2% BSA in TBSN overnight at 4°C. Glands were incubated with 50x dilution of primary antibody in PBS and 2% BSA for 8 hours. Glands were washed with TBSN for a

further 48 hours with buffer washes every 8-12 hours before incubation with the appropriate FITC-coupled secondary antibody in TBSN, 2% BSA for 8 hours at 4°C in the dark. Glands were washed in TBSN for 48 hours before dehydration in a graded series of methanol solutions (0-100%) over 90 minutes. Glands were mounted on microscope slides in clearing solution and viewed with a Zeiss LSM-410 confocal laser scanning microscope and Zeiss Own™ imaging software.

3.5.5. DEGENERATIVE PRIMER DESIGN

The NCBI (National Centre for Biotechnology Information) SwissProt database was searched for known SNARE proteins and serine protease amino acid sequences. The Clustal W Program was used to create multiple alignments of the homologous amino acid sequences in order to identify conserved areas (Thompson *et al.* 1994). The homologous regions were used as templates to design a primer sequence using the Oligo Version 4.0 program (National Biosciences, USA)(Rychlik and Rhoades 1989). Inosine was included at positions of high redundancy. The T_m was calculated by the equation: $69.3 + 0.041 \times (\% \text{ G/C}) - (650 / \text{length})$ (Rychlik *et al.* 1990).

3.5.6. TOTAL RNA ISOLATION

Total RNA was isolated from either unfed (200 glands) or fully engorged (30 glands) female *O. savignyi* salivary glands. Ticks were fed to completion (30-45 minutes) on New Zealand white rabbits and dissected within 2 hours. Glands were transferred to 750 µl TRI-REAGENT® (Molecular Research Center, INC). Total RNA was isolated according to the manufacturer's instructions up to the step where the total RNA fraction was in the aqueous fraction. The RNA was not precipitated immediately as suggested, but loaded onto a RNA isolation column (RNeasy®, Qiagen) for further purification. The column was washed according to the instructions and the RNA eluted in 50 µl DEPC-treated water. The purity and quantity of RNA was determined spectrophotometrically by measuring the 260/280 nm ration and the 260 nm value, respectively. RNA quality was assessed by denaturing agarose gel electrophoresis on a 1% agarose gel prepared in 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA and 18% formamide in DEPC treated double distilled deionized water. Electrophoresis was conducted at 70 V for 30 minutes.

3.5.7. CONVENTIONAL cDNA SYNTHESIS

Single strand cDNA was prepared using Superscript™ II (Life Technologies) and the anchor-dT primer (Joubert *et al.* 1998). Total RNA (1 µg) in 7.5 µl DEPC treated water was denatured at 70°C for 3 minutes and snap cooled on ice. Five pmoles of anchor-dT primer (1 µl), 3 µl DTT (0.1 M), 200 units Superscript™ II (1 µl), 4 µl 5x first strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂, pH 8.3) was added and incubated at 42 °C for 60 minutes before inactivation at 70 °C for 2 minutes. The cDNA was stored at – 70°C.

3.5.8. SUPER SMART™ cDNA SYNTHESIS

The Super SMART™ PCR cDNA synthesis kit (Qiagen) provides a novel, PCR-based method for producing high-quality cDNA from nanogram quantities of total RNA. A modified oligo(dT) primer, the 3' CDS Primer IIA primes the first-strand synthesis reaction (Figure 3.6). When the MMLV (Moloney murine leukemia virus) reverse transcriptase (RT) reaches the 5' end of the mRNA, the enzyme's terminal transferase activity adds a few (up to 5) additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA. The SMART™ oligonucleotide, which has an oligo(G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template. RT then switches templates and continues replication to the end of the oligonucleotide. The resulting full-length, single-stranded cDNA contains the complete 5' end of the mRNA as well as sequences that are complementary to the SMART™ oligonucleotide. The SMART™ anchor sequence and the poly(A) sequence serve as universal priming sites for end-to-end cDNA amplification. In contrast, cDNA without these sequences, such as prematurely terminated cDNAs, contaminating genomic DNA or cDNA transcribed from poly(A)- RNA will not be exponentially amplified (Clontech Laboratories 2001a). Since the terminal transferase activity of the RT adds a random number of deoxycytidine to the first strand, directional cloning and expression of these fragments are possible since various reading frames were generated randomly.

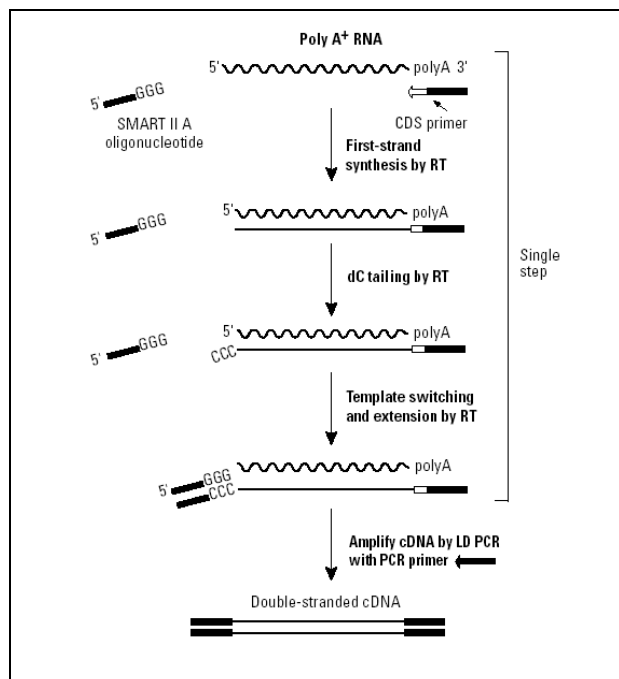


Figure 3.6. Flow chart of Super SMART™ cDNA synthesis (Clontech Laboratories 2001a).

First strand cDNA synthesis was performed by combining 500 ng total RNA, 7 μ l of the 3' SMART CDS III primer (12 μ M), 7 μ l SMART IV primer (12 μ M) and water up to a final volume of 64 μ l. See Table 3.10 for primer properties. The contents were mixed with a pipette and collected by brief centrifugation. The RNA was denatured at 65°C for 2 minutes and then kept at 42°C. 20 μ l of 5x First-strand buffer, 2 μ l DTT (100 mM), 10 μ l 50x dNTP (10 mM), 2.5 μ l RNase inhibitor (40 U/ μ l), 2.5 μ l SuperScript II RNase H- reverse transcriptase (200 U/ μ l) and 5 μ l water were added. The mixture was mixed by gently pipetting and then centrifuged briefly. Since full-length cDNAs were required for downstream applications, the mixture was incubated for 90 minutes at 42°C. Finally the reaction was terminated with the addition of 2 μ l of 0.5 M EDTA.

To purify the SMART cDNA from unincorporated nucleotides and small (<0.1 kb) cDNA fragments, the cDNA was subjected to column chromatography using the NucleoSpin® Extraction kit (Machery Nagel, Germany). Three volumes of buffer NT2 (details of buffer composition withheld by supplier) were added to each cDNA synthesis reaction, mixed by pipetting and loaded onto the column. The column was centrifuged at 13 000 x g for 1 minute and the flow through discarded. The column was washed three times with 500 μ l buffer NT3 (details of buffer composition withheld by supplier) and centrifuged (13 000 x g, 1 minute). In order to remove any remaining ethanol, the column was placed in a clean tube

and again centrifuged. Water (50 μ l) was added to the column filter and soaked for 2 minutes before eluting the cDNA into a clean tube with centrifugation. The elution process was repeated with 35 μ l water using the same collection tube. The total recovered elution volume was 80-85 μ l per sample. The cDNA was stored at -70°C until used.

3.5.9. cDNA amplification by LD-PCR

Based on the guidelines provided by Clontech, 5 μ l of ss cDNA was used for cDNA amplification by long distance PCR (LD-PCR). The ss cDNA was diluted in 79 μ l water to which 10 μ l of ExTaq buffer, 2 μ l 50x dNTP mix (10 mM) and 2 μ l 5' PCR Primer II (12 μ M) was added (see Table 3.10 for primer properties). The mixture was mixed well by pipetting and centrifuged briefly. The tube was placed in a preheated thermal cycler and incubated for 1 minute at 94°C before lowering the temperature to 80°C and adding 2 μ l ExTaq polymerase (5 U/ μ l). PCR cycling was performed at 95°C for 15 seconds, 65°C for 30 seconds and elongation at 68°C for 6 minutes. After 15 cycles, 30 μ l was removed for optimization of the number of PCR cycles. The remaining 70 μ l was stored at 4°C and 25 μ l of the optimization fraction returned to the thermal cycler. After every 3 cycles, 5 μ l was removed up to a final of 30 cycles. The 5 μ l fractions were analyzed on a 1.2% agarose / ethidium bromide gel alongside DNA molecular size markers. The optimal number of cycles required for each sample was determined as one cycle fewer than is needed to reach the plateau, ensuring that the ds cDNA remains in the exponential phase of amplification. The remaining 70 μ l was returned to the thermal cycler and subjected to the number of additional cycles. Usually, 24 cycles were optimal for 500 ng of salivary gland cDNA. Finally the ds cDNA was purified using the NucleoSpin extract kit (as described earlier) and the yield determined at 260 nm on the Gene Quant Pro system (Biochrom Ltd., Cambridge, Supplied by Amersham Biosciences). On average 1,8 -2,2 μ g of SMART cDNA was obtained after purification.

3.5.10. RANDOM AMPLIFICATION OF 3' cDNA ENDS (3'-RACE)

Both single stranded cDNA obtained after normal cDNA synthesis or ds cDNA obtained from SMART cDNA synthesis were used during 3'-RACE. In all cases, the optimal concentrations of cDNA, primers, MgCl_2 , annealing temperature and number of cycles were optimized for every reaction using a Taguchi optimization matrix (Cobb and Clarkson 1994). Single-stranded cDNA (0.1- 0.5 μ l) or SMART ds cDNA (50 ng) were combined with 10 pmol of the appropriated anchor primer, 5 - 50 pmol of the degenerative primer, 2 μ l TaKaRa Taq™ PCR

buffer, 1 - 1.5 mM MgCl₂, 200 μM dNTP's and water to a final volume of 20 μl. The cDNA was denatured at 94°C for 3 minutes and then cooled to 80°C after which 5 μl of enzyme mix (2.5 U TaKaRa Taq diluted in 1x PCR buffer) was added. Amplification consisted of 21-30 cycles of DNA denaturation (94°C, 30 seconds), annealing (at various temperatures depending on the T_m of the degenerative primer used, 30 seconds) and extension (72°C, 2 minutes) followed by a final extension step (72°C, 5 minutes). All amplification procedures were conducted in a Gene Amp PCR system 9700 (Perkin Elmer Applied Biosystems). The principle of 3'-RACE using degenerative primers is depicted in Figure 3.7.

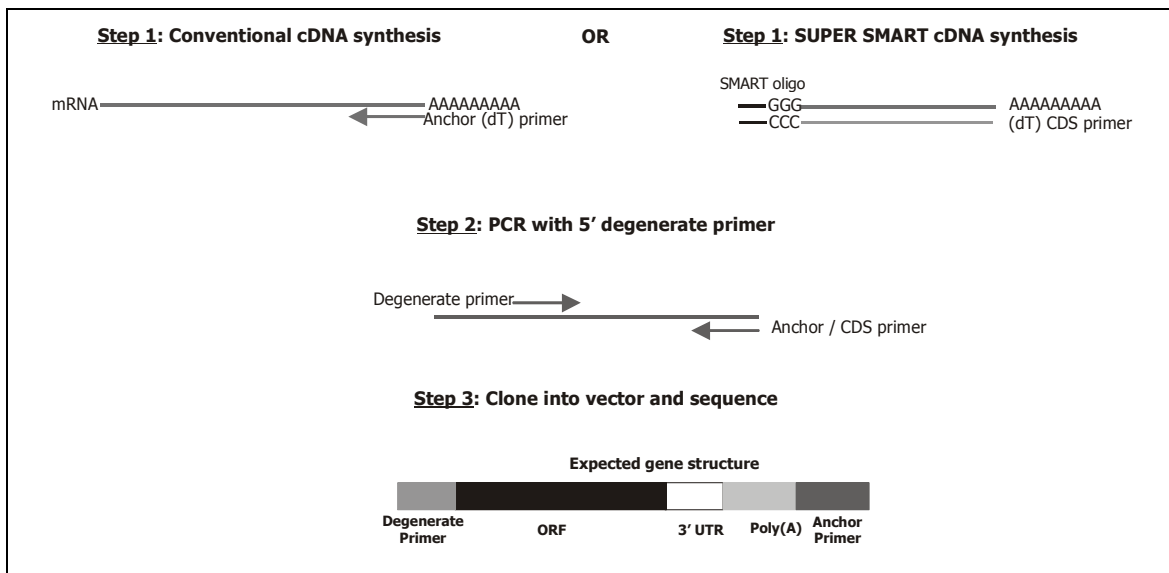


Figure 3.7. Cloning strategy during 3'-RACE (adopted from Mans, 2002). (1) Single stranded cDNA was synthesized with an anchor (dT) primer and ds cDNA with the (dT) CDS and SMART primers. (2) A degenerate primer and an anchor primer was used to amplify the product of interest. (3) The product was cloned into the pGEM T-easy vector (Promega) and sequenced.

3.5.11. DIG-LABELLING OF PROBES USING PCR

The synaptotagmin I probe was synthesized from the recombinant sytI plasmid obtained from Prof. Scheller. In order to use the T3 and T7 primers, the coding region of sytI was cloned into Bluescript. The PCR mixture consisted of 2 μl Takara Taq DNA polymerase (5 U/μl), 2 μl dNTPs, 0.25 nmol DIG-dUTP, 1.5 mM MgCl₂, 5 pmol of each primer, and 0.5 μl of template (1:10 000 dilution of 50 ng/μl stock) in a total volume of 20 μl. Twenty-three cycles were done with an annealing temperature of 50°C for 30 seconds, denaturation at 94°C for 30 seconds and extension at 72°C for 2 minutes.

3.5.12. DNA DOT BLOTTING

DNA was denatured for 10 minutes at 95°C and chilled immediately on ice to prevent re-annealing. DNA samples (1 μ l, typically 10-100 ng) were spotted onto a Nylon membrane (Roche Molecular Biochemicals), allowed to dry and exposed to UV-light (Spectroline transilluminator, Spectronics Corporation, New York, USA, $\lambda=312$ nm) for 3 minutes to cross-link the DNA to the membrane. The membrane was placed in a hybridization bag containing standard hybridization solution (5x Sodium citrate saline solution (SSC), 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent) and incubated at 65°C for 2 hours. The probe was diluted to a concentration of 25 ng/ml in standard hybridization solution and after removing the pre-hybridization buffer the membrane was incubated overnight at 65°C with the probe. Excess probe was removed by washing the membrane twice for 15 minutes in 2x washing solution (2x SSC, 0.1% SDS) and twice for 15 minutes in 0.5x washing solution (0.5x SSC, 0.1% SDS) at 65°C. The membrane was equilibrated in detection wash buffer (100 mM maleic acid, 150 mM NaCl, 0.3% Tween 20, pH 7.5) for 1 minute and then blocked in the detection buffer containing 1% blocking reagent for 60 minutes at 25°C. Following the blocking step, the membrane was incubated with anti-digoxigenin alkaline phosphatase Fab fragments (1:20,000 dilution in blocking buffer) for 30 minutes at 37°C and again washed twice for 15 minutes with detection wash buffer at 37°C to remove unbound Fab fragments. The membrane was equilibrated for 2 minutes in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl) and incubated for 5 minutes in the CDP-Star™ substrate (diluted 1:100 in detection buffer). Exposing the membrane to X-ray film was used for the detection of the chemiluminescent signal.

3.5.13. AGAROSE GEL ELECTROPHORESIS

PCR products were analyzed by electrophoresis at 68 V using a 1, 1.5 or 2% analytical grade agarose gel (D-1 Low EEO Agarose, Conda, Spain). TAE (40 mM Tris, 1mM EDTA) was used as electrophoresis buffer in a mini gel apparatus (Biometra, GmbH). Molecular masses were calculated from the Rf-values of the molecular mass markers (Promega, Wisconsin, USA).

3.5.14. PCR PRODUCT PURIFICATION

PCR products were purified from either agarose gels or a PCR reaction mixture. In both cases, the NucleoSpin® Extract kit (Machery Nagel, Germany) was used. During purification from agarose gels, gel pieces were dissolved in 300 μ l buffer NT1 and incubated at 50°C until the gel slices were dissolved (5-10 min). The sample was loaded onto a NucleoSpin®

extract column, centrifuged (8000 x g, 1 min) and the flow through discarded. The column was washed with 600 µl buffer NT3 and centrifugation (11 000 x g, 1min) and then with 200 µl NT3 (11 000 x g, 2min). DNA was eluted in 40 µl elution buffer (5 mM Tris-HCl, pH 8.5) after centrifugation (11 000 x g, 1min). The procedure for isolation from PCR reactions is identical, except that the gel-dissolving step is omitted and the sample is diluted in 4 volumes of buffer NT2.

3.5.15. QUANTIFICATION OF NUCLEIC ACIDS

All DNA and RNA samples were quantitated spectrophotometrically using the Gene Quant Pro™ system (Biochrom Ltd., Supplied by Amersham Biosciences) using a mini cuvette (10 µl).

3.5.16. A/T CLONING OF PCR PRODUCTS INTO pGEM® T-EASY VECTOR

DNA products were ligated into 50 ng of the pGEM® T-Easy vector (Promega) at a 3:1 insert to vector ratio. Ligation was performed at 4°C overnight with 1 µl T4 ligase and 5 µl 2x ligation buffer of the pGEM® T-Easy vector system (Promega, USA). Prior to transformation, the T4 ligase was inactivated at 70 °C for 10 minutes.

3.5.17. PREPARATION OF ELECTROCOMPETENT CELLS

A single colony of SURE or BL21 *E. coli* was inoculated into 15 ml Luria-Berthani Broth (LB Broth, 1% NaCl, 1% Tryptone, 0.5% yeast extract in deionized water, pH 7.4) and grown overnight at 30°C with moderate shaking. Two 5 ml fractions of the overnight culture were diluted into 2 x 500 ml of pre-warmed LB broth in a sterile 2-liter flask, and grown at 37°C with vigorous shaking until OD₆₀₀ reached 0.5 to 0.6. The cultures were transferred into four pre-chilled 250 ml centrifuge bottles, placed on ice for 20 minutes and then centrifuged (10 000 x g, 20 min, 4°C). The cell pellet was suspended in 10 ml ice cold H₂O with swirling, another 240 ml of ice cold water was added and the cells were collected by centrifugation (10 000 x g, 20 min, 4°C). After another three washes with 250 ml water, the cell pellets were dissolved in 10 ml glycerol (10% v/v), pooled into two sterile 50 ml centrifuge tubes, incubated on ice for 60 minutes and again centrifuged to collect the cells. Finally, cell pellets were suspended in a total volume of 1 ml glycerol (10% v/v) and divided into 90 µl aliquots that were stored at -70°C.

3.5.18. TRANSFORMATION BY ELECTROPORATION

To each ligation reaction, one-tenth volumes of tRNA (10 mg / ml) and sodium acetate (3 M, pH 5) as well as three volumes of 100% ethanol were added. The mixture was centrifuged (13 000 x g, 30 minutes, 4°C) to collect the plasmid-tRNA complex. The precipitate was washed with 1 ml 70% ethanol, centrifuged (13 000 x g, 10 min, 4°C) and dried *in vacuo*. The pellet was dissolved in 10 µl water and added to 90 µl electro-competent cells, which were thawed on ice. The complete 100 µl mixture was placed into a pre-chilled 0.1 cm gap electroporation cuvette and electroporated at 2.5 - 3 kV for 5 milli-seconds. Immediately after electroporation, the cells were removed from the cuvette, diluted in 1 ml LB-broth and incubated at 30°C with moderate shaking for 60 minutes before plating 10-50 µl of transformed cells onto 1.5% agar that contained 50 µg/ml ampicillin, 40 µg/plate X-gal (20 mg/ml DMSO) and 4 µl/plate IPTG (400 mM). Positive colonies were selected using blue-white selection with conventional plasmid isolation and restriction enzyme digestion of plasmid to identify inserts with the correct size.

3.5.19. MINIPREP PLASMID ISOLATION

Positive colonies were grown overnight in a shaking incubator at 30 °C in LB broth containing ampicillin (10 µg/ml). The cells of 2 ml culture were collected by centrifugation (12 000 x g, 10 minutes) and resuspended in 100 µl solution I (25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA, pH 8). Cells were subsequently lysed with the addition of 150 µl of solution II (200 mM NaOH, 1% SDS), mixed by gentle inversion and incubation at room temperature for 10 minutes. Chromosomal DNA and SDS were removed with the addition of 250 µl of solution III (3 M potassium acetate, pH 4.2) and incubation on ice for 10 minutes. The insoluble precipitate was removed by centrifugation (13 000 x g, 15 minutes, 4°C). Plasmid DNA was precipitated from the supernatant with the addition of 1 ml cold, absolute ethanol and centrifugation (13 000 x g, 30 minutes, 4°C). The pelleted plasmid was washed with 1 ml cold, 70% ethanol and again centrifuged. The pellet was dried using the Bachoffer vacuum concentrator and dissolved in 30-50 µl water.

3.5.20. HIGH PURE PLASMID ISOLATION

High purity plasmids were isolated by means of the High Pure™ plasmid purification kit (Roche Diagnostics). Cells from 2 ml of culture were isolated by centrifugation (13,000 x g, 2 minutes, 4°C) and suspended in 250 µl suspension buffer (50 mM Tris-HCl, 10 mM EDTA, 0.1 ml/ml RNase, pH 8). Cells were lysed at room temperature for 5 minutes with the

addition of 250 μ l lysis buffer (200 mM NaOH, 1% SDS). Afterwards, 350 μ l binding buffer (4 M guanidine hipochloride, 0.5 M potassium acetate) were added to precipitate chromosomal DNA and increase the ionic strength for sufficient binding to the silica matrix. Chromosomal DNA was removed by centrifugation (12 000 x g, 15 minutes, 4°C) and the supernatant loaded onto the High Pure filter tube, which contain the silica filter for binding of DNA in the presence of chaotropic salts. The solution was centrifuged (12 000 x g, 1 min) and the flow through discarded. The column was washed twice. First with 700 μ l wash buffer I (5M guanidine hydrochloride, 20 mM Tris-HCl, pH 6.6, 40% ethanol) and then 700 μ l wash buffer II (2 mM Tris-HCl, 20 mM NaCl, 80% ethanol, pH 7.5). In order to remove any residual ethanol, the filter was dried by centrifugation (12,000 x g, 2 minutes). The High Pure filter tube was transferred to a clean tube and plasmid eluted with 100 μ l elution buffer (1 mM Tris-HCl, pH 8.5) and centrifugation (12 000 x g, 2 minutes).

3.5.21. AUTOMATED DNA SEQUENCING AND DATA ANALYSIS

Sequencing was performed with the Big Dye Sequencing kit (Perkin Elmer, Foster City) on an ABI Prism 377 DNA sequencer (Perkin Elmer Applied Biosystems, USA). Each reaction contained 2 μ l Ready reaction mixture™, 3 μ l 5x buffer (400 mM Tris-HCl, 10 mM MgCl₂, pH 9), 1 μ l primer (3.2 pmol), 300 ng plasmid and water to a final volume of 20 μ l. PCR sequencing was performed using 25 cycles of denaturation (96°C, 10 seconds), annealing (50°C, 5 seconds) and extension (60°C, 4 minutes). The mixture was transferred to a siliconized tube before precipitation of the PCR product with the addition of 64 μ l absolute ethanol and centrifugation (13,000 x g, 20 minutes, 4°C). The pellet was washed with 100 μ l 70% ethanol, centrifuged, dried and dissolved in 3 μ l loading dye (5:1 ratio of deionized formamide and 25 mM EDTA, pH8 and blue dextran, 30 mg/ml). Samples were denatured at 95°C (2 minutes), cooled on ice and analyzed.

Sequences obtained were analyzed using the BioEdit Program. DNA and deduced protein sequences were analyzed using BLAST-P, PSI-BLAST (www.ncbi.nlm.nih.gov/BLAST) and the threading program 3D-PSSM Web Server V 2.6.0 (www.igb.uci.edu/tools/scratch/). All alignments were performed with Clustal W (www.ebi.ac.uk/clustalw/).

3.6. RESULTS AND DISCUSSION

3.6.1. Western blotting of salivary glands with anti-SNARE and anti-Rab3a antibodies

By means of polyclonal antibodies against the three core proteins of the fusion machinery, we were able to detect a syntaxin, SNAP25 and VAMP homologue in the salivary glands of *O. savignyi* (Figure 3.8). The molecular masses of the identified proteins correspond to those in literature for both the ixodid and brain proteins (Karim *et al.* 2002). In all cases the proteins were enriched in the membrane fractions (lanes 2 and 4) obtained after centrifugation. Syntaxin was also detected in the supernatant fractions.

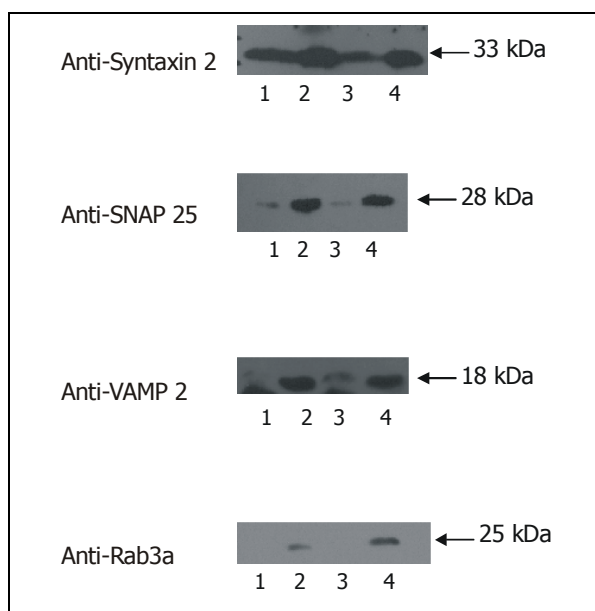


Figure 3.8. Identification of SNAREs and Rab3a using Western Blotting. Salivary glands were homogenized and fractionated by centrifugation. The supernatant (lane 1) and pellet (lane 2) obtained after 2000 \times g as well as the supernatant (lane 3) and pellet (lane 4) obtained after 100,000 \times g was analyzed.

Prior to fusion, SNARE tethering complexes are formed between syntaxin, SNAP25 and VAMP. These complexes are SDS-resistant and can be identified by comparing boiled and non-boiled samples during Western blotting. The presence of a high molecular mass complex in *O. savignyi* was investigated by using membrane fractions obtained after 100,000 \times g centrifugation and anti-VAMP2 polyclonal antibodies. As a positive control, homogenized rat brain was subjected to centrifugation (100,000 \times g) and the pellet fraction was analysed. From the results (Figure 3.9) we identified the rat brain 20S complex corresponding to 78 kDa (lane 1) and a high molecular mass complex (81 kDa) in the pellet of *O. savignyi* (lane

3). In both the boiled and non-boiled fractions obtained from *O. savignyi*, the monomeric VAMP (18 kDa) is visible, although it is enriched in the boiled fraction (lanes 2 and 3). This indicates that unlike brain tissue (lane 1), not all of the VAMP-proteins are present in complexes at one time. In both lanes 1 and 3 a second high molecular mass VAMP-containing complex is visible which could be the VAMP-synaptophysin complex described in literature (Edelman *et al.* 1995) since a synaptophysin homologue was also identified in *A. americanum* (Karim *et al.* 2002).

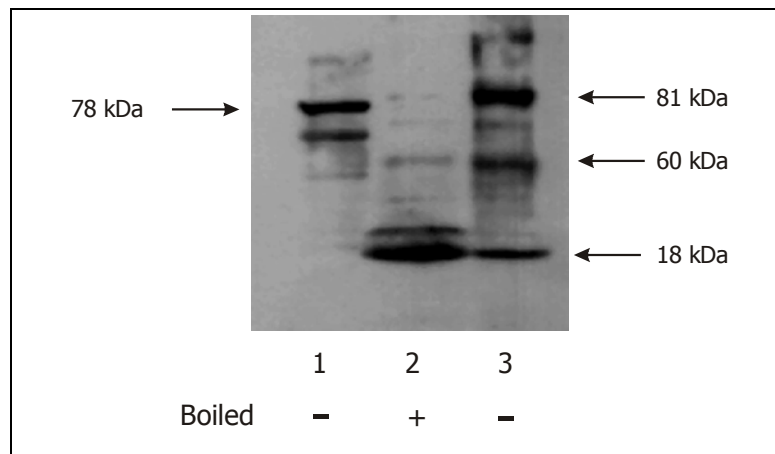


Figure 3.9. Identification of a high molecular mass core complex in the salivary glands of *O. savignyi*. Western Blot analysis using anti-VAMP2 of non-boiled rat brain pellet (lane 1), boiled (lane 2) and non-boiled (lane 3) *O. savignyi* salivary gland pellet fractions obtained after centrifugation at 100,000 x g.

The data obtained during Western blotting confirmed the presence of the core fusion proteins syntaxin, VAMP and SNAP25, as well the core fusion complex formed by these three proteins. Apart from the SNARE proteins, we also identified a Rab3a GTPase homologue in the salivary glands of *O. savignyi*.

3.6.2. Localization of SNAREs and cytoskeleton proteins using confocal microscopy

Syntaxin

Syntaxin isoforms 1A, 1B, 2, 3 and 4 are all located on the plasma membrane of cells (Teng *et al.* 2001). By using anti-syntaxin 2 polyclonal antibodies and a FITC-coupled secondary antibody, we were able to localize a syntaxin-2 homologue to the cell plasma membrane of *O. savignyi* (Figure 3.10). No intra-cellular syntaxin was detected.

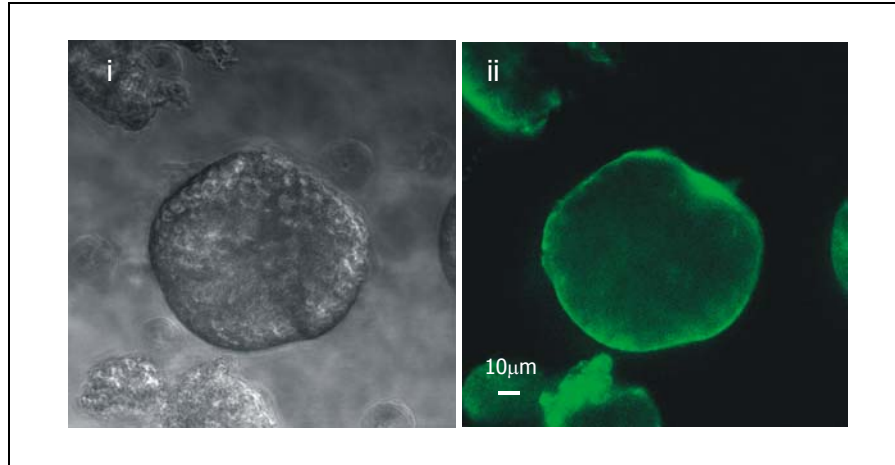


Figure 3.10. Immuno-localization of syntaxin in the salivary glands of *O. savignyi* using anti-rat brain syntaxin2 polyclonal antibodies. Light microscopy (i) and fluorescence confocal image (ii) of a granular salivary gland cell.

VAMP

Immuno-localization of VAMP using anti-rat brain VAMP2 polyclonal antibodies indicated VAMP to be localized to the granule membrane, since individual granules are visible (Figure 3.11.ii and iii). From Figure 3.11.iii it is evident that VAMP does not localize to the plasma membrane itself. In some acini, cells containing small granules enriched in VAMP were identified (Figure 3.11.iii). The identity of these small granules is unknown, but based on the size we hypothesize that these correspond to small granules (1-2 μm) of the 'c' cells as described previously (Chapter 2) and B.J. Mans (Mans 2002a).

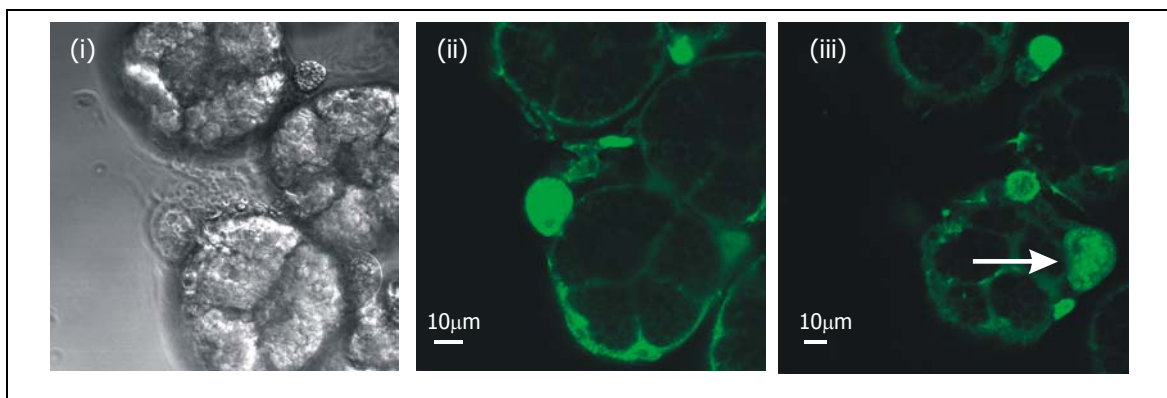


Figure 3.11. Immuno-localization of VAMP in the acini of *O. savignyi* using anti-rat brain VAMP2 polyclonal antibodies. Light microscopy (i) and fluorescence confocal image (ii and iii) of granular salivary gland acini. The arrow in figure iii indicates the VAMP2 enriched small granules.

Immuno-localization of VAMP in granular cells indicated VAMP to again localize to the granule membranes (Figure 3.12). In order to determine if VAMP is present on the plasma membrane, we performed 3D-scanning confocal microscopy. The results indicated that VAMP is not present on the plasma membrane of granular cells (Figure 3.12.ii).

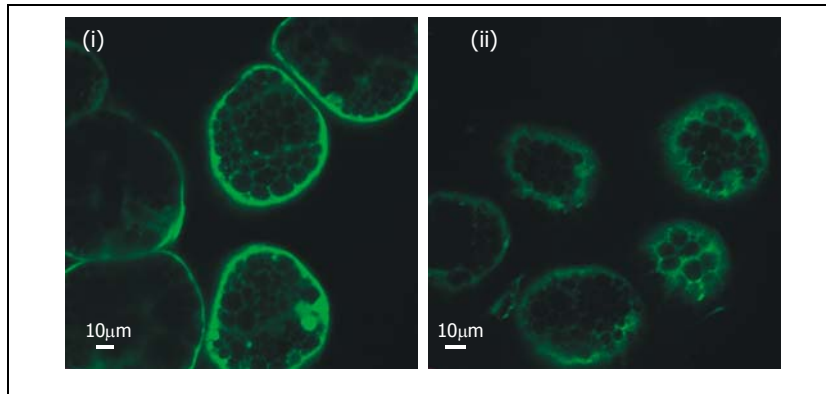


Figure 3.12. Immunofluorescence images of *O. savignyi* salivary glands. VAMP localizes to granule membranes (i), but not the cell plasma membrane (ii).

SNAP 25

Immunofluorescence localization of SNAP25 indicated small granules/vesicles enriched in SNAP25 (Figure 3.13.i). In acini, localization of SNAP25 to granule membranes was confirmed since individual granules are visible (Figure 3.13.ii).

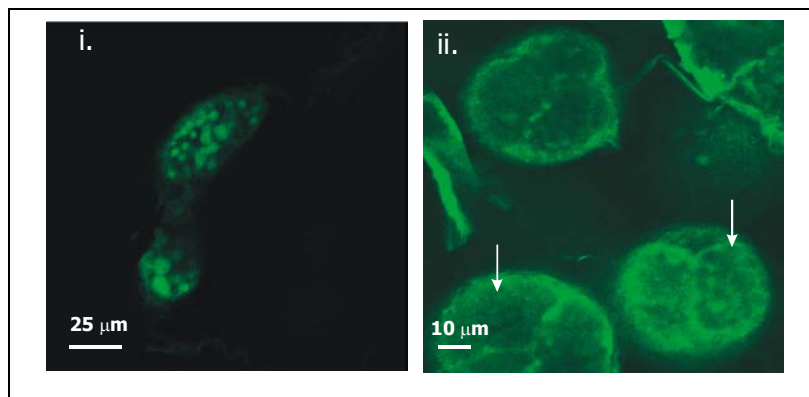


Figure 3.13. Immunofluorescence images of *O. savignyi* acini. SNAP25 was localized to small granules (i) and granule membranes (ii). Arrows indicate individual granules.

Cytoskeleton proteins: Actin and Tubulin

Both of the cytoskeleton proteins actin and tubulin play important roles during exocytosis. The actin cytoskeleton is believed to regulate exocytosis by forming a cortical actin network

beneath the plasma membrane and to also mediate the transport of secretory granules to exocytotic sites on the plasma membrane (Burgoyne and Morgan 2003). Immuno-localization of actin in granular cells of *O. savignyi* indicated actin to localize predominantly to the sub-plasma membrane region, supporting the presence of an actin barrier in tick salivary glands (Figure 3.14). Actin is also visible between the granules since individual granules are visible.

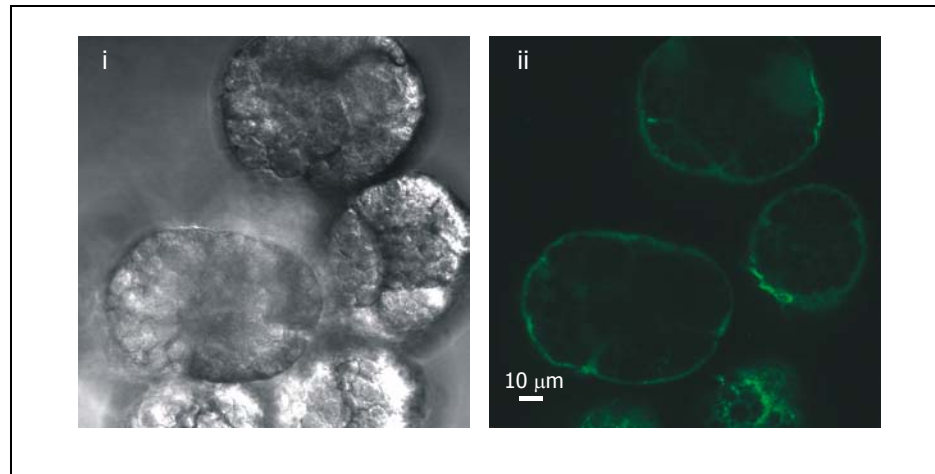


Figure 3.14. Immuno-localization of actin in acini of *O. savignyi*. Actin localized to the plasma membrane region in granular acini. Both the light microscope image (i) and the fluorescence confocal image (ii) are shown.

Similar to actin, tubules are required for the transport and delivery of proteins throughout the entire secretory pathway (Apodaca 2001; Neco *et al.* 2003). Localization of tubulin in acini was indicated to the sub-plasma membrane of the cells, as well as the intracellular tissue between the granules (Figure 3.15). Therefore, one can conclude that tick salivary glands do contain an extensive cytoskeletal structure and that it is involved in exocytosis (see Chapter 2).

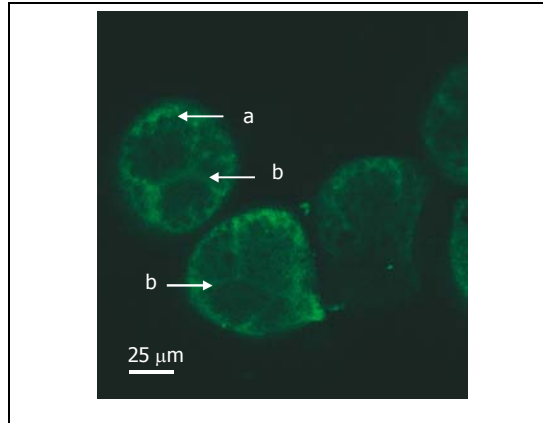


Figure 3.15. Immuno-localization of tubulin in the acini of *O. savignyi*. Tubulin localized to either the (a) intra granular spaces or (b) the sub-plasma membrane region of granular cells.

3.6.3. RNA isolation

Analysis of the total RNA isolated from the salivary glands of *O. savignyi* indicated only a 18S rRNA band, which is different from total RNA isolated from tissues containing 28S, 18S and 5S rRNA units. This observation has been described previously (Joubert *et al.* 1998; Mans 2002a). In contrast, RNA isolated from whole *O. savignyi* ticks (all tissues excluding salivary glands) and fed *Argas (P.) walkerae* larvae contained the 28S, 18S and 5S rRNA bands (Figure 3.16.ii and iii).

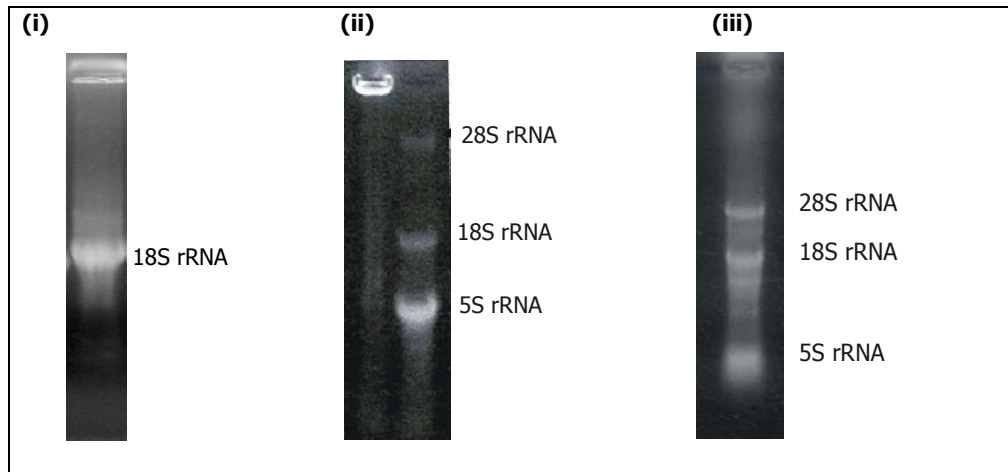


Figure 3.16. Electrophoretic analysis of total RNA. Figures corresponds to total RNA isolated from (i) salivary glands of *O. savignyi*, (ii) the remaining tissues of *O. savignyi*, and (ii) whole *Argas (P.) walkerae* larvae.

3.6.4 3'-RACE USING ss cDNA

cDNA was synthesized as described previously using the method of Joubert *et al.*, which employs an anchor-dT primer and reverse transcriptase (Joubert *et al.* 1998). 3'-RACE was subsequently performed with a degenerative primer and anchor primer (Figure 3.7).

Synaptotagmin

Based on the techniques used to date to identify synaptotagmin isoforms from various tissues (Table 3.6), we decided on exploiting a synaptotagmin I (sytI) probe as well as degenerative primers. The entire coding sequence of sytI was amplified from the recombinant sytI construct obtained from Prof. R.H. Scheller, using PCR and sequence specific primers. A single band corresponding to 975 bp was obtained (Figure 3.17). Identical conditions were used to amplify the sytI band in the presence of DIG-dUTP to create a DIG-labelled probe. The probe was purified using the NucleoSpin[®] Extract kit.

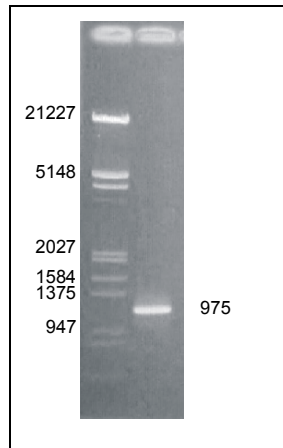


Figure 3.17. Agarose electrophoresis of the open reading frame amplified from recombinant synaptotagmin I.

Degenerative primers were designed on two conserved regions identified in synaptotagmin isoforms I, II, III, IV and V (Figure 3.18). The first sequence (FDRFS) is present in the PKC-C2A domain while the second sequence (SDPYVK) is present in both PKC-C2 (A and B) domains. The properties of the degenerative primers are listed in Table 3.7.

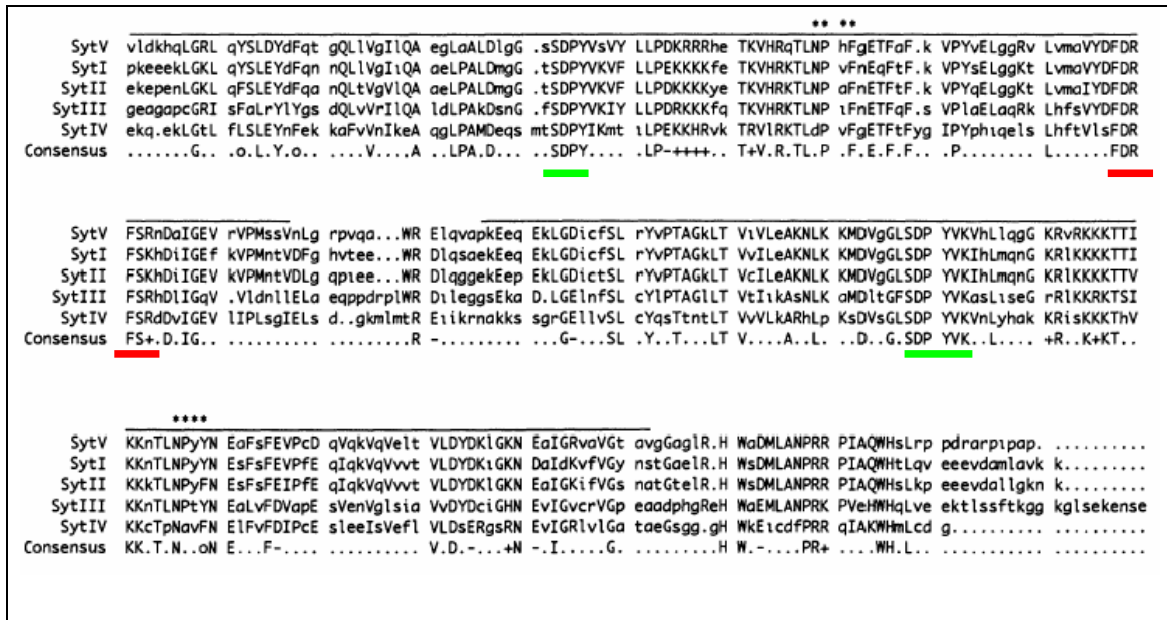


Figure 3.18. Amino acid similarity among five synaptotagmin isoforms (Hudson and Birnbaum 1995). Amino acid sequences of the five synaptotagmin isoforms are aligned. Conserved and identical amino acids are capitalized. A consensus sequence appears on the bottom, depicting only amino acids that are identical among all isoforms. In the consensus sequence, completely conserved acidic or basic residues are indicated with a (-) or (+), respectively, and conserved aromatic residues are indicated by (o). The PKC-C2 domains are overlined with a solid line while the NPXY sequence is denoted by asterisks (X indicates any amino acid).

Table 3.7. Properties of the synaptotagmin degenerative primers. Degenerate nucleotide nomenclature corresponds to: (W) is A or T, (N) is any nucleotide, (R) is A or G, (S) is G or C, (M) is A or C and (Y) is C or T.

Name	Sequence	Degeneracy	Tm (°C)
Syt_1 (SDPYVK)	WST GAY CCT TAY GTN AAR	128	48.77
Syt_2 (FDRFS)	TTY GAY TTY GAY MGT TT	32	45.4

Using the Syt_1 primer we were able to identify various bands during PCR using cDNA from both salivary glands and whole *O. savignyi* ticks (Figure 3.19). As a control, cDNA from rat brain was used. The expected size was 735 bp for the open reading frame of rat brain sytI (excluding the 3' UTR and polyA tail). In lanes 11-13 an intense band was observed at ~800 bp for the brain samples, while two bands (~950 and 1100 bp) were observed for the *O. savignyi* samples.

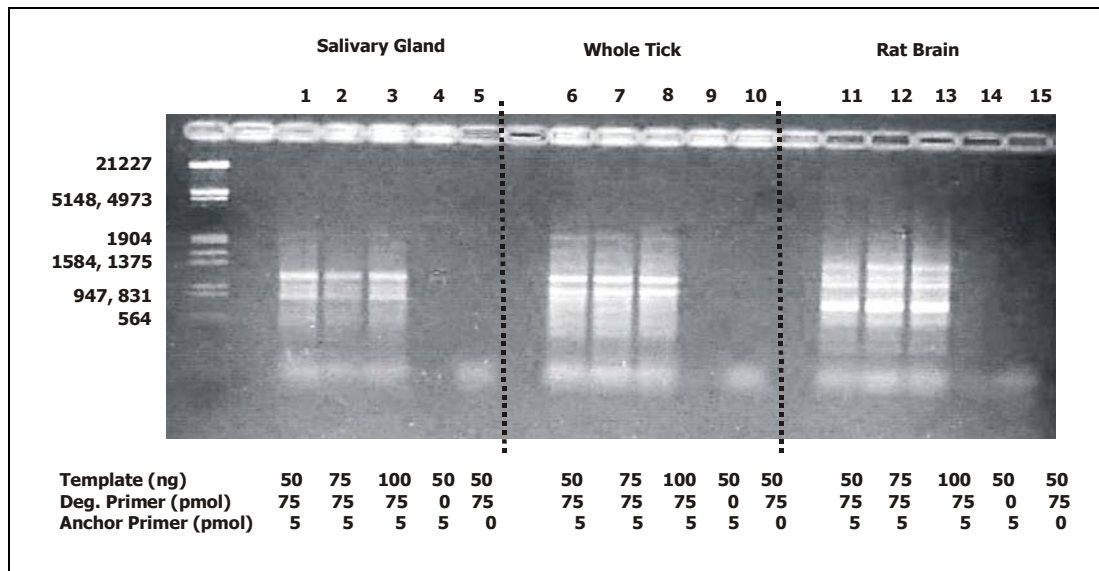


Figure 3.19. 3'-RACE with synaptotagmin primer 1 (SDPYVK) and cDNA created from salivary glands of *O. savignyi*, whole *O. savignyi* ticks and rat brain (positive control). The conditions used during 3'-RACE are listed for each sample.

Since the PCR could not be optimized to obtain only single specific bands, the bands were excised, purified and cloned into the pGEM-T easy vector. Using restriction enzyme mapping unique clones were identified. These were grown, subjected to high pure plasmid isolation and hybridization with the sytI probe. All positive clones were subjected to DNA sequencing. The results obtained from all of the *O. savignyi* samples indicated no similarity to synaptotagmin (results not shown). Synaptotagmin was, however, successfully cloned and identified from the rat brain samples.

3'-RACE performed with the syt_2 primer yielded a single band of ~500 bp (Figure 3.20). Once again the rat brain cDNA, which was used as positive control, yielded a similar mass band (~600 bp). The various bands were purified, cloned and screened via restriction enzyme digestion and hybridization with the sytI probe (Figure 3.21). All unique positive clones were subjected to DNA sequencing. Once again, no similarity to synaptotagmin was obtained for the tick clones (results not shown).

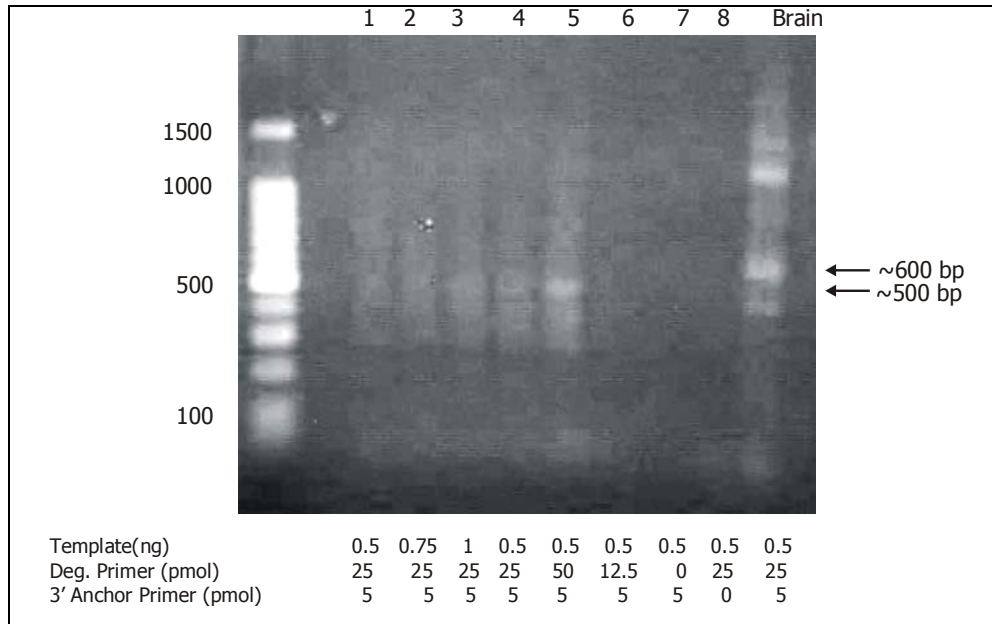


Figure 3.20. 3'-RACE with salivary gland RNA and the *syt_2* primer. *The conditions used during 3'-RACE are listed for each sample.*

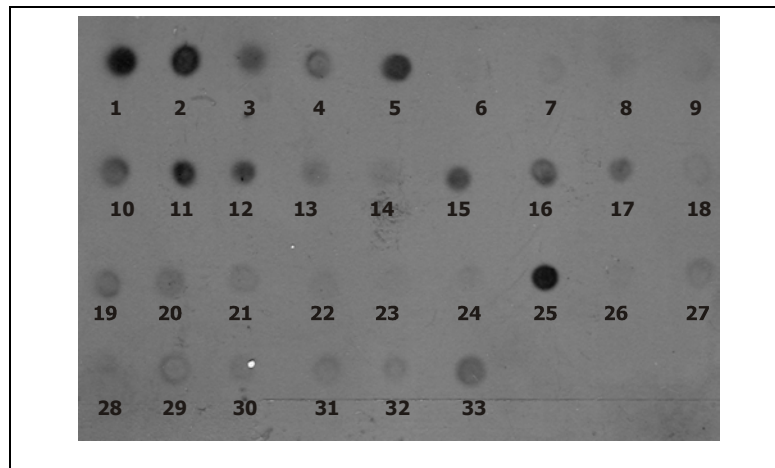


Figure 3.21. Hybridisation of the putative synaptotagmin clones obtained with the DIG-labelled *sytI* probe.

Syntaxin

Amino acid sequence alignment of various syntaxin isoforms from various diverged organisms indicated five conserved regions (Figure 3.22). The RKFVEVM and VESQGEM regions were used for designing degenerative primers for 3'-RACE due to the lower degeneracy of the codons encoded for in these amino acid sequences. The properties of the two primers are listed in Table 3.8.

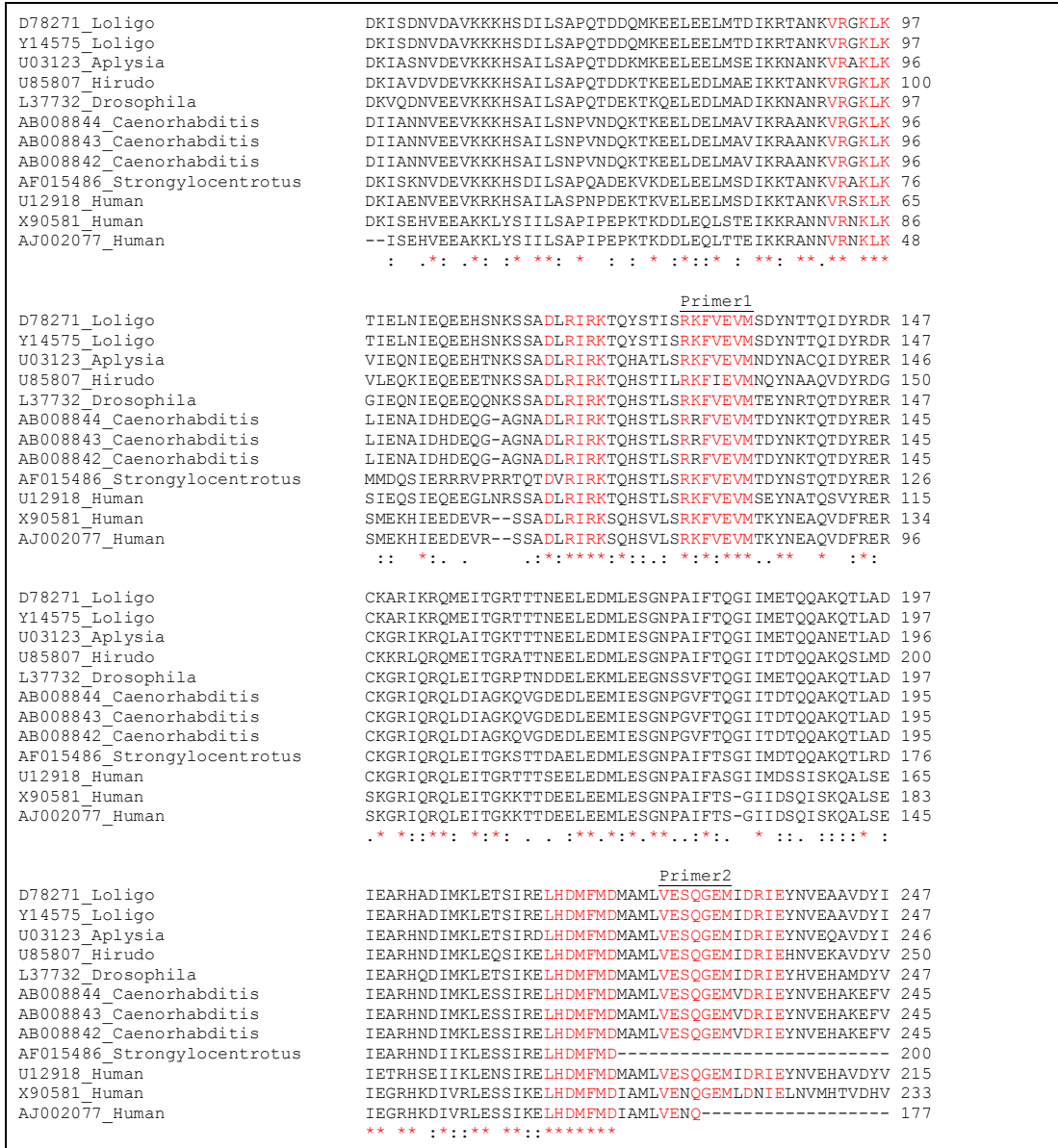


Figure 3.22. Amino acid sequence alignment of various syntaxins. Sequences were obtained from the NCBI databank. Identical (*), similar charge (:), and similar polarity (.) of residues are indicated. Conserved regions indicated in red were considered for degenerative primer design.

Table 3.8. Properties of the syntaxin degenerative primers. Degenerate nucleotide nomenclature corresponds to: (W) is A or T, (N) is any nucleotide, (R) is A or G, (S) is G or C, (Y) is C or T and (M) is A or C.

Name	Sequence	Degeneracy	Tm (°C)
Syn_1 (VESQGEM)	GTI GAR WSI CAR GGN GAR ATG	128	58.0
Syn_2 (RKFVEVM)	MGI AAR TTY GTI GAR GTN ATG	64	53.0

PCR amplification using the syn_1 degenerative primer and cDNA from the salivary glands of *O. savignyi* identified a single band of ~700 bp, which is in the expected size region. This band was however not cloned since the PCR was driven only by the degenerative primer and not both the degenerative and anchor primers (Figure 3.23). PCR amplification using cDNA obtained from fed *Argas walkerae* larvae (another argasid tick used as control) yielded a single band, which was amplified by both primers (Figure 3.24). This band was purified, cloned and subjected to DNA sequencing. The obtained nucleotide and protein sequence is given in Figure 3.25.

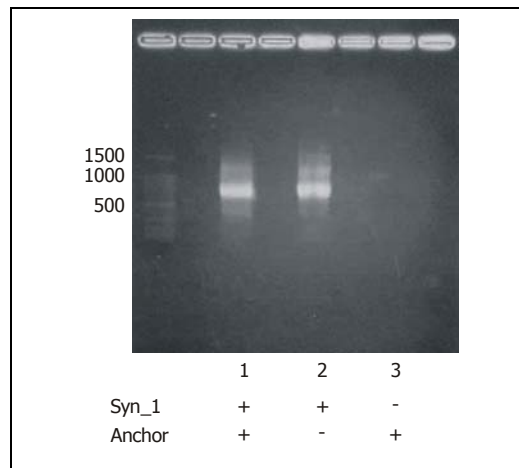


Figure 3.23. PCR amplification of syntaxin using the syn_1 degenerative primer from *O. savignyi* salivary gland cDNA.

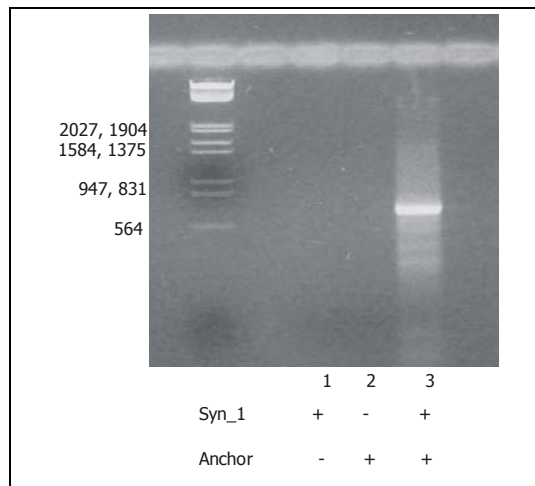


Figure 3.24. PCR amplification of syntaxin using the syn_1 degenerative primer from *Argas (P.) walkerae* cDNA.

```

gtggagtggcagggcgagatggttgagagggccaggaagctgtctgactactttccatct
V E W Q G E M F E R P G K L S D Y F P S
ccataccccaatgaagaggcagcaagagcagcgaataatgggtgctcttccacctgacctc
P Y P N E E A A R A A N N G A L P P D L
agcttcatcacgaatgctaatacatggcggagaggactacatcttccgactggtgacggga
S F I T N A K H G G E D Y I F A L L T G
tactgcgatccaccgcaggagtgcagatccaagaagggtcagtactacaaccctacttc
Y C D P P A G V T I Q E G Q Y Y N P Y F
ccagggggagctatcgccatggccaagctcttacaatgaggccatggaatactctgat
P G G A I G M A Q A L Y N E A M E Y S D
ggcacaccggccactactagtcagatggcaaaggatgtactactttcctcaggttctgca
G T P A T T S Q M A K D V L L S S G S A
cggagccanagtttgatgaccgcaaacgcatgtttatcaagggcatgatgatctgtcact
R S X S L M T A N A C L S R A - -
gctgcttgn tacgacctgttacttgn aaccggcagaatggatgac

```

Figure 3.25. DNA nucleotide and amino acid sequence of *A. walkerae* clone obtained with syn_1. The primer region is indicated in red and the stop codon (-) is visible.

Both BLAST_P and PSI-BLAST analysis of the deduced protein sequence indicated a significant similarity to the cytochrome C1 heme protein as well as the cytochrome C1 precursor, with E-values of 2×10^{-41} and 3×10^{-39} , respectively. Threading analysis indicated 53% identity to the sequence and structure of the cytochrome C1 transmembrane subunits.

PCR amplification using the syn_2 primer, based on the amino acid sequence RKFVEVM, was optimized using a Taguchi optimization matrix (Cobb and Clarkson 1994). The three conditions that were varied were the $MgCl_2$ concentration, syn_2 primer concentration and amount of template (cDNA/RNA equivalents). A single ~550 bp band was obtained under numerous conditions (Figure 3.26). This band was purified (Figure 3.27), cloned and subjected to DNA sequencing. The nucleotide and deduced amino acid sequence is given in Figure 3.28. Both BLAST-P and PSI-BLAST analysis were unable to detect any possible identity of the encoded protein. Threading indicated similarity to DNA binding proteins (25%), various collagen isoforms (25-26%) and insect defensins (28%). Therefore, degenerative primers and 3'-RACE was not successful in amplifying a syntaxin homologue from *O. savignyi*.

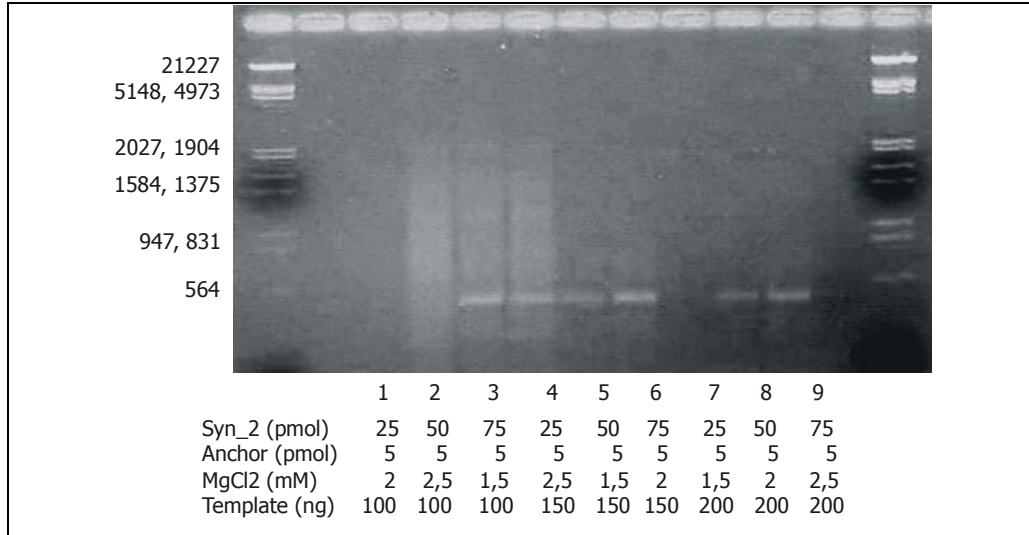


Figure 3.26. Taguchi-PCR with syn_2 using salivary gland cDNA from *O. savignyi*. The conditions used during 3'-RACE are listed for each sample.

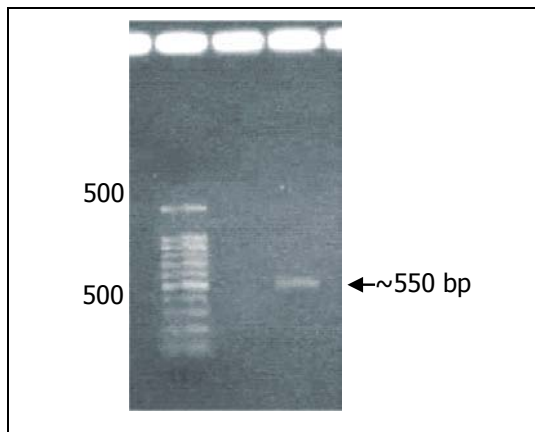


Figure 3.27. High Pure isolation of the 500 bp band obtained with syn_2.

```

aggaaattcgtggaggttatgtatggttacgcacggtctcagtgtaagcttaagagcacc
R K F V E V M Y V T H G L S V S L R A P
catctaccgctacctgggacttcgaggtgtgtggcatgcacgagatgcattggagaaaat
H L P L P G T S R C V A C T R C I G E N
caaatgagatgacgttgattagtgaggttacatcagttgactgcgattgatttagctta
Q N E M T L I S E V T S V D C D - F S L
cacagattttgtttctttgttagttgcaccaatgacaacgtatggcctactacatcgc
H R F L F L C L V A P M T T Y G L L H R
gtatcccgcctacgcactgtactgtatggtgccgaaattctgcaatgacatgtacgtcgat
V S R Y A L Y C M V P K F C N D M Y V D
agggtacagctgcgtgcacttcgcggttgttcagaaattgggtattaacagggtatctaaa
R V Q L R A L R V V Q K L G I N R V S K
gcagcatgtacgactccacagtgaagaaaatactccgcgtatgtcgagattggtaaatga
A A C T T P Q -
ggaacatgacttaattgtacggaaaaaaaaaaaaa
    
```

Figure 3.28. Nucleotide and amino acid sequence of the 500 bp band obtained with syn_2 primer from salivary gland cDNA. The primer sequence is indicated in purple. The stop codon (-) and poly(A) tail is visible.

Serine protease (Positive control)

As a positive control, we designed a primer against possible high abundance salivary gland proteins such as serine proteases. The serine proteases are divided into two families: the trypsins and subtilisins. The trypsin family is the largest and is found in vertebrates, as well as fungi and prokaryotic cells (<http://www.diapharma.com>). A degenerative primer was designed against the most conserved region (GDSGGPL), which was identified from aligned serine protease amino acid sequences. The properties of the primer are given in Table 3.9.

Table 3.9. Properties of the serine protease degenerative primer. *Degenerate nucleotide nomenclature corresponds to: (W) is A or T, (N) is any nucleotide, (S) is G or C and (Y) is C or T.*

Name	Sequence	Degeneracy	Tm (°C)
Ser1 (GDSGGPLV)	GTG AYW STG GTG GTC CIY TIG TN	64	61.5

Initial PCR studies using total RNA from unfed *O. savignyi* salivary glands revealed only a smear and no distinct bands could be observed. After optimization, two bands (~200 and 300bp, respectively) were obtained. DNA sequencing and analysis of the bands indicated that none of the bands displayed significant similarity to any known protein. These results indicated that (i) the transcript is not present in the cDNA pool, or (ii) the transcript is present in very low abundance, or (iii) the primer is not suitable for the identification of a serine protease. These conclusions also applied to the previously described PCRs for the identification of synaptotagmin and syntaxin. Therefore, we decided to firstly use RNA from fed ticks in which transcription is enhanced and secondly, to equalize the cDNA using suppression PCR.

3.6.5 3'-RACE USING SUPER SMART™ ds cDNA

RNA was isolated from fully engorged ticks. There was an approximate 3.5 fold increase in tick weights. During ds DNA synthesis using the Super SMART™ principle, only a single primer is used during LD-PCR, since the 5' sequence (obtained from the SMART IV primer) and the 3' sequence (obtained from the CDS III primer) are complementary (Table 3.10). Therefore, during PCR amplification of the ds DNA, intra-molecular annealing events can result in the formation of panhandle-structures (Figure 3.29), which will not be amplified. This is more likely to occur in high abundance transcripts, allowing the exponential amplification of low abundance transcripts and hence establishing a representative cDNA library.

Table 3.10. Super SMART™ primers used for cDNA synthesis and LD-PCR.

Name	Sequence
SMART IV	AAG CAG TGG TAT CAA CGC AGA GTG GCC ATG GAG GCC GGG
CDS III	ATT CTA GAG GCC TCC ATG GCC GAC ATG T ₃₀ VN
3' PCR	ATT CTA GAG GCC TCC ATG GCC GAC ATG
5' PCR	AAG CAG TGG TAT CAA CGC AGA GT

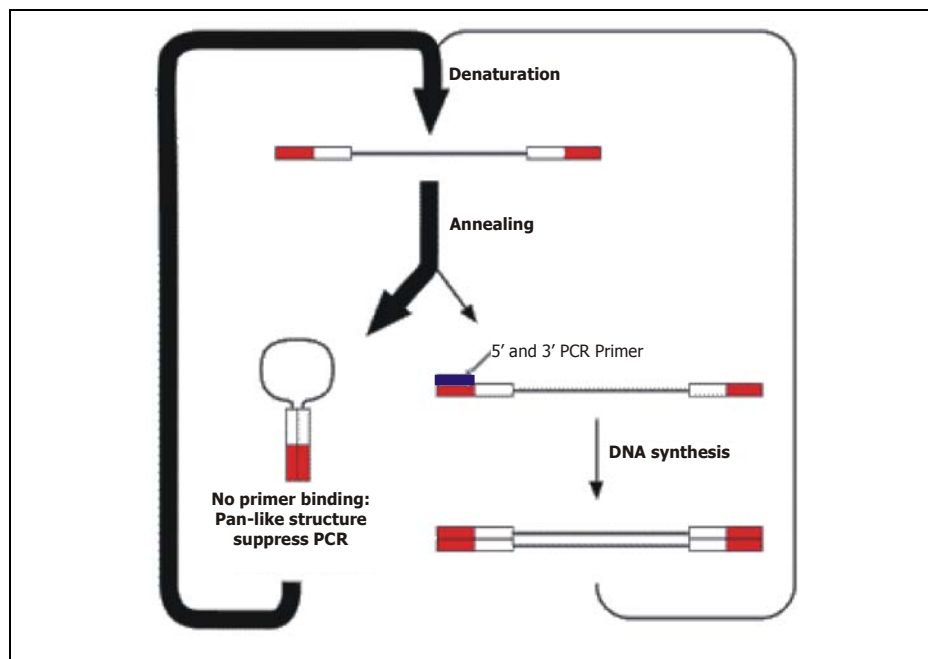
**Figure 3.29. Schematic presentation of the suppression PCR effect. Adapted from (Lukyanov *et al.* 1997).**

Figure 3.30 shows a typical gel profile of ds cDNA synthesized using the Super SMART™ cDNA synthesis and amplification system. The optimal amount of cycles (1 cycle less than needed to reach the plateau) was determined as 25 for both the salivary gland and whole tick (include all tissues and organs except salivary glands) RNA samples. A smear between 200-2000 bp was obtained from salivary gland RNA, while a 200-5000 bp smear was obtained from the RNA isolated of the remaining tissues of *O. savignyi* (Figure 3.30). Following amplification, the ds DNA was purified using the NucleoSpin® kit and used for 3'-RACE.

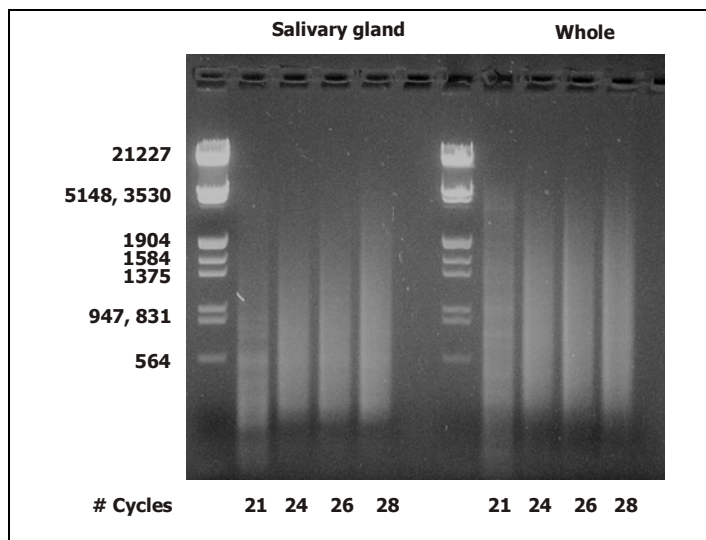


Figure 3.30. Analysis of ds cDNA amplification by LD-PCR using Super SMART™ technology. The number of cycles used to determine the plateau is indicated for each sample.

3' - RACE using SuperSMART™ ds DNA

During 3'-RACE using SuperSMART ds DNA as template, the identical degenerative primers as described previously were used, but the anchor primer was replaced by the 3' PCR primer (Table 3.10).

Serine protease (Positive control)

3'-RACE using the serine protease degenerative primer yielded two bands. These were purified, cloned and sequenced. BLAST analysis of the inserts indicated similarity to serine proteinase-1 from *Rhipicephalus appendiculatus* with a significant E-value of 1×10^{-6} , as well as serine proteases from bovine, rat and human origin. This indicates that the degenerative primer was correctly designed and that the previous inability to identify the correct transcript was most probably due to low abundance or absence of the mRNA transcript.

Syntaxin

Taguchi optimisation of the 3'-RACE conditions using the syn_1 primer indicated a prominent band at ~ 450 bp and a smear (Figure 3.31). Optimization of the conditions resulted in the amplification and purification of the 450 bp band (Figure 3.32). This band was cloned and sequenced.

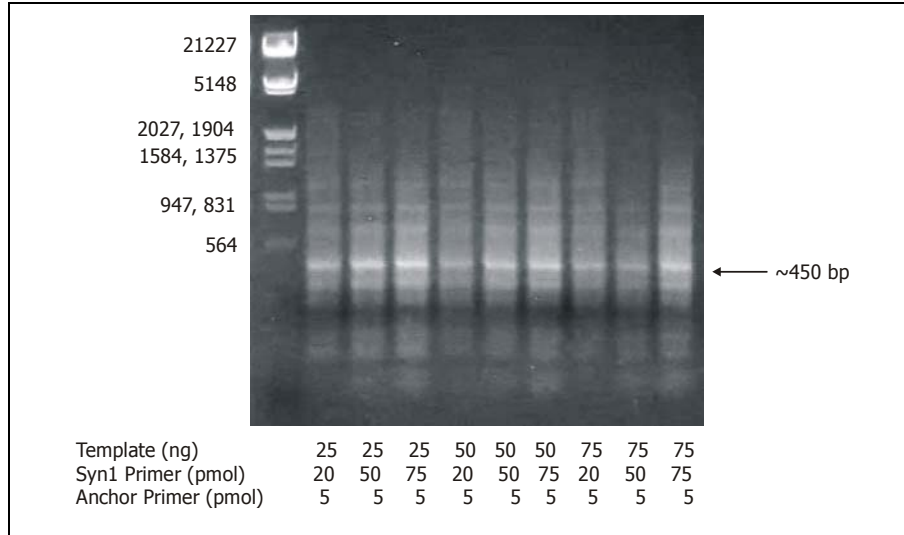


Figure 3.31. Taguchi_PCR with the syn_1 primer using ds SMART DNA from salivary glands of *O. savignyi*. The various conditions used during 3'-RACE are listed for each sample.

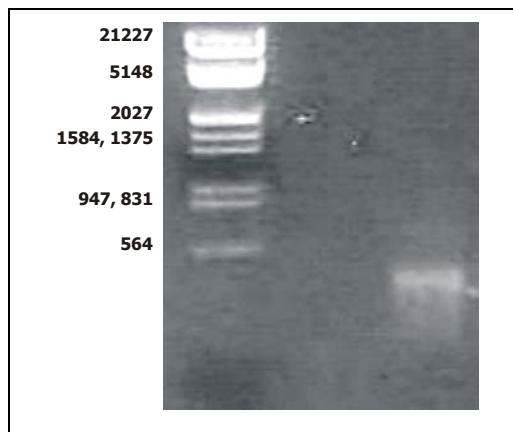


Figure 3.32. Agarose electrophoresis of the purified 450 bp product obtained with syn_1 and SMART DNA.

```

gtggagtggcagggggagatgaaagacaaggcggaaatcctcttcnctacgaggaacag
V E W Q G E M K D K A E I L F X Y E E Q
tctcaagtcaaagatgaacttactcaagaggttgagagctgcacgccgattagagcaa
S Q V K D E L T Q E V G E L H A A L E Q
gaacgttccaaggtccattccttgcaaacggagctcaagaagccaggcaaaagggacaac
E R S K V H S L Q T E L K K P G K R D N
cggtgatggtggcagccccctatttttttcaagtcattttggccagtcttccaaaggatc
R -
tggagacagtattaacgggaacaggatccagaagctttccttcattttgtatatagcagc
tgtgggataaaattcatttccggcagaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
    
```

Figure 3.33. Nucleotide and amino acid sequence of 450bp band obtained with syn_1 primer from SMART salivary gland cDNA. The primer region is indicated in red. The stop codon (-) and poly(A) tail is visible.

BLAST-P and PSI-BLAST analysis of the encoded protein identified highest similarity to the GKAP-42 protein (E-value: 8×10^{-11}) and protein kinase anchoring proteins (E-value: 1×10^{-9}). Threading identified various proteins, all belonging to the all-alpha protein class, such as the Eps15 homology domain and the RAP domain of alpha-2-macroglobulin receptor associated protein. Again, no similarity to syntaxin was detected.

Syntaxin isoforms 2 and 3

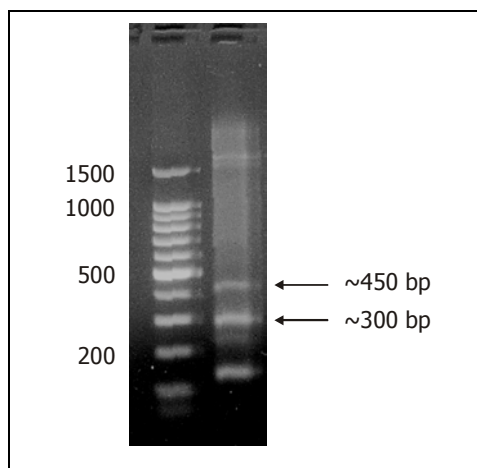
In order to investigate whether tick syntaxins share sequence similarity to isoforms other than syntaxin 1, we aligned mouse, rat, human and rabbit syntaxin 2 and 3 isoforms (Figure 3.34). Various conserved regions suitable for degenerative primer design were identified. The less degenerative primer could be designed against the MLESG-region and the properties of the primer are listed in Table 3.11. During 3'-RACE, two distinct bands were obtained at 450 and 350 bp, respectively. These bands were purified, cloned and subjected to DNA sequencing.

MusEpim	SIILSAPNPEGKIKEELEDLDKEIKKTANRIRGKLKLSIEQSCDQDENGNR TSV DLRIRRT	118
Rat_2A	SIILSAPNPEGKIKEELEDLNKEIKKTANRIRGKLKKAIEQSCDQDENGNR TSV DLRIRRT	119
Rat_2C	SIILSAPNPEGKIKEELEDLNKEIKKTANRIRGKLKKAIEQSCDQDENGNR TSV DLRIRRT	119
Rat_2B	SIILSAPNPEGKIKEELEDLNKEIKKTANRIRGKLKKAIEQSCDQDENGNR TSV DLRIRRT	119
EPIM_Homo	SIILSAPNPEGKIKEELEDLNKEIKKTANKIAAKLKAIEQSFQDDESGNR TSV DLRIRRT	117
Rat_3A	SIILSAPIPEPKTKDDLEQLTTEIKKRANNVRNKLKSMKHEIEDE--VRSSADLRIRKS	118
Human_3	SIILSAPIPEPKTKDDLEQLTTEIKKRANNVRNKLKSMKHEIEDE--VRSSADLRIRKS	118
Orycto_3	-----DDLEQLTTEIKKRANNVRNKLKSMERHIEIEDE--VRSSADLRIRKS	44
	:**:* :*** **.: ****::: ::* *:*.*****:~	
	Primer	
MusEpim	QHSVLSRKFF DVMTEYNEAQILFRERSKGR RIQR Q LEIT GR TTDDE ELEEMLESGKPSIFI	178
Rat_2A	QHSVLSRKFF DVMTEYNEAQILFRERSKGR RIQR Q LEIT GR TTDDE ELEEMLESGKPSIFI	179
Rat_2C	QHSVLSRKFF DVMTEYNEAQILFRERSKGR RIQR Q LEIT GR TTDDE ELEEMLESGKPSIFI	179
Rat_2B	QHSVLSRKFF DVMTEYNEAQILFRERSKGR RIQR Q LEIT GR TTDDE ELEEMLESGKPSIFI	179
EPIM_Homo	QHSVLSRKFF EAMAEYNEAQTFRERSKGR RIQR Q LEIT GR TTDDE ELEEMLESGKPSIFT	177
Rat_3A	QHSVLSRKFF EVMTKYNEAQVDFRERSKGR RIQR Q LEIT GK TTDDE ELEEMLESGNPAIFT	178
Human_3	QHSVLSRKFF EVMTKYNEAQVDFRERSKGR RIQR HVEITGK TTDDE ELEEMLESGNPAIFT	178
Orycto_3	QHSVLSRKFF EVMTKYNEAQVDFRERSKGR RIQR Q LEIT GK TTDDE ELEEMLESGNPAIFT	104
	.**.:*:* ** :*:*.*.*****:*****:***:****:*****:~**	
MusEpim	SDIISDSQITRQALNEIESRHKDIMKLETSIRELHEMFMDMAMFVETQGEMVNNIERNVV	238
Rat_2A	SDIISDSQITRQALNEIESRHKDIMKLETSIRELHEMFMDMAMFVETQGEMVNNIERNVV	239
Rat_2C	SDIISDSQITRQALNEIESRHKDIMKLETSIRELHEMFMDMAMFVETQGEMVNNIERNVV	239
Rat_2B	SDIISDSQITRQALNEIESRHKDIMKLETSIRELHEMFMDMAMFVETQGEMVNNIERNVV	239
EPIM_Homo	SDIISDSQITRQALNEIESRHKDIMKLETSIRELHEMFMDMAMFVETQGEVINNIERNVM	237
Rat_3A	SGII-DSQISKQALSEIEGRHKDIVERLESSIKELHDMFMDIAMLVENQGEMLDNIELNV	237
Human_3	SGII-DSQISKQALSEIEGRHKDIVERLESSIKELHDMFMDIAMLVENQGEMLDNIELNV	237
Orycto_3	SGII-DSQISKQALSEIEGR-----	123
	..* ****:~**.* ** *	

Figure 3.34. Amino acid sequence alignment of various syntaxin isoforms 2 and 3. Sequences were obtained from the NCBI databank. Identical (*), similar charge (:), and similar polarity (.) of residues are indicated. Conserved regions indicated in yellow were considered for degenerative primer design.

Table 3.11. Properties of the syn_2/3 degenerative primer. Degenerate nucleotide nomenclature corresponds to: (W) is A or T, (N) is any nucleotide, (R) is A or G, (S) is G or C and (Y) is C or T.

Name	Sequence	Degeneracy	Tm (°C)
Syn_2/3 (MLESGK)	GAR GAT ATG YTT GAR WSN GG	96	58

**Figure 3.35. 3'-RACE with the syn_2/3 primer using ds SMART DNA from salivary glands of *O. savignyi*.**

The nucleotide sequence was analysed in all three reading frames in order to determine the encoded protein sequence (Table 3.12). BLAST-N of the nucleotide sequences indicated no homology to any known protein. PSI-BLAST and P-BLAST analysis of the amino acid sequences also indicated no homology to any known proteins. Threading indicated the highest similarity to neurotoxins such as the omega-conotoxin mvia2 as well as microbial and mitochondrial isozyme 2.

Table 3.12. Amino acid sequence of the proteins encoded for in the 450 bp and 300 bp bands amplified with the syn_2/3 primer.

Name	Amino acid sequence
450 bp Band	DMFERGQCGRGYSSRSRSTRSTKATSTLPEGRSLRRRRGPLPFQGWRSaipvslagLQVMVSLKTHCSATLVY
300 bp Band	DELEDMLASTNMWKSkrlllrchrtpkrs

We concluded that the SNAREs and secretory proteins in *O. savignyi* do not share sufficient sequence homology (to known isoforms) to such an extent that degenerative primers can be used. Therefore, other methods that exploit the structural properties of SNARE proteins, such as protein-protein interactions (see Chapter 4), functional complementation in knockout

yeast and affinity chromatography (see Chapter 5), must be exploited in order to isolate the conserved exocytotic machinery in *O. savignyi*.

3.7. CONCLUSION

By means of polyclonal antibodies directed at rat brain SNAREs, we were able to identify homologues of syntaxin, VAMP and SNAP25 in *O. savignyi* salivary glands. Therefore one can conclude that structural similarity exists between rat, ixodid- and argasid tick isoforms. The localization of the SNARE homologues in the salivary glands of unfed *O. savignyi* is summarized in Figure 3.36.

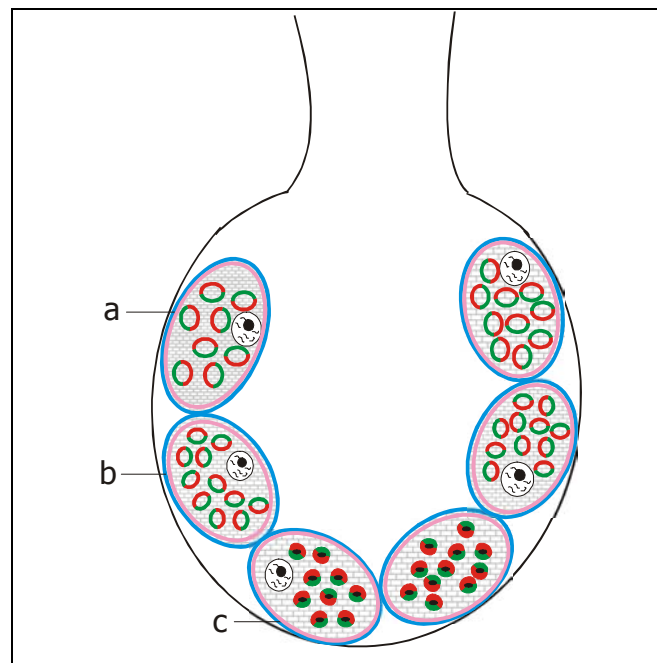


Figure 3.36. Localization of SNAREs and cytoskeletal proteins in the acini of *O. savignyi*. Syntaxin (Blue), SNAP25 (red), VAMP (green), actin (pink) and tubulin (grey background) are indicated. We hypothesize that the small vesicles enriched in both VAMP and SNAP25 are located to the 'c' cells (filled-circles), and the granule membranes of 'a' and 'b' cells (open circles).

3'-RACE with degenerative primers were used in an attempt to isolate the various SNARE and secretory protein transcripts from *O. savignyi*. RNA was successfully isolated from the salivary glands of some 100 unfed ticks and converted to cDNA using a poly(T)-anchor primer. As positive control, cDNA created from total rat brain RNA was used. In the latter case, synaptotagmin could be amplified and correctly identified. We also exploited a synaptotagmin I probe, which identified possible positive clones incorrectly in *O. savignyi*.

Possible reasons for not detecting SNAREs or secretory proteins could include: (a) mRNA synthesis is only initiated upon feeding, and (b) the low abundance of the mRNA transcripts.

Therefore, *O. savignyi* ticks were fed to completion on rabbits before isolating total RNA from some 30 glands. Double stranded, cDNA was synthesized from as little as 500 ng total RNA by using the Super SMART system (Clontech). The latter protocol involves equalization of the transcripts by means of suppression PCR. As a positive control, a primer against a high abundance transcript (encoding serine proteases) was used. By means of 3'-RACE, we were able to successfully amplify and clone a trypsin-like serine protease. Studies using the degenerative primers directed against the various SNAREs and secretory proteins were unsuccessful.

Our data indicates that the structural properties of *O. savignyi* secretory proteins does resemble that of rat brain, but since the degenerative primers were unsuccessful in amplifying SNAREs, one can conclude that the nucleotide sequences differ significantly between tick SNAREs and the consensus sequences used for primer design. Alignments of all known syntaxins to date indicated no significant conserved regions useful for primer design amongst the entire family. Therefore, methods exploiting the functional and structural characteristics of the secretory proteins such as protein-protein interactions (Chapter 4), functional complementation in knockout cells (Chapter 5) and affinity (Chapter 5) will be exploited in order to isolate these proteins.

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