

CHAPTER 1

LITERATURE REVIEW

1.1. TICKS: AN OVERVIEW

Ticks are believed to have originated 120 million years ago (MYA), and that they speciated by ~92 MYA into the main tick families as we know them today (Mans 2002a). Currently, they are classified as belonging to the subclass Acari, the dominant subclass of the Arachnida. The approximately 850 species are divided into two major and one small subfamily. The first family is the Ixodidae or 'hard ticks', so called because of their sclerotized dorsal scutal plate. The second family is the Argasidae or 'soft ticks', so called because of their flexible, leathery skin/cuticle. A third family, the Nuttalliellidae, contains only one species, *Nuttalliella namaqua* (Sonenshine 1991).

Ixodidae

The family Ixodidae is by far the largest and economically most important family. It contains 13 genera and approximately 650 species (Sonenshine 1991). They are characterized by the presence of a tough, sclerotized plate (scutum) on the dorsal body surface, which functions as the site of attachment for various muscle groups (Figure 1.1).

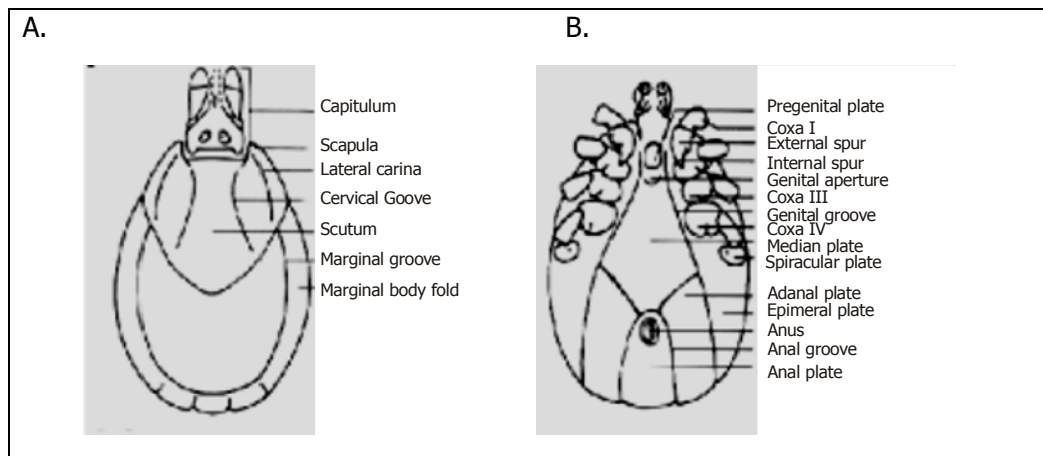


Figure 1.1. Diagram illustrating ixodid adult tick body structures (Sonenshine 1991). The (A) *Female ixodid tick, dorsal aspect, and (B) male, ventral aspects are shown.*

In males, the scutum covers the entire dorsal surface, limiting the expansion of the body during feeding. In females, nymphs and larvae, the scutum is limited to the anterior dorsal body region and allows for significant expansion of the body during feeding. In fully

engorged specimens, the scutum appears as a small plate on an extended body. Anterior to the scutum, the mouthparts protrude beyond the body and are visible when the specimens are viewed from the dorsal aspect (Sonenshine 1991).

During the life cycle of Ixodidae (Figure 1.2), eggs hatch into a single nymphal instar, which will molt to larvae. The nymphal and larval stages resemble adults, but lack the external genital pores, porose areas and foveal pore clusters. In larvae, only three pairs of walking legs occur, with the fourth pair represented by limb buds that develop into legs with the molt to the nymphal state (Sonenshine 1991).

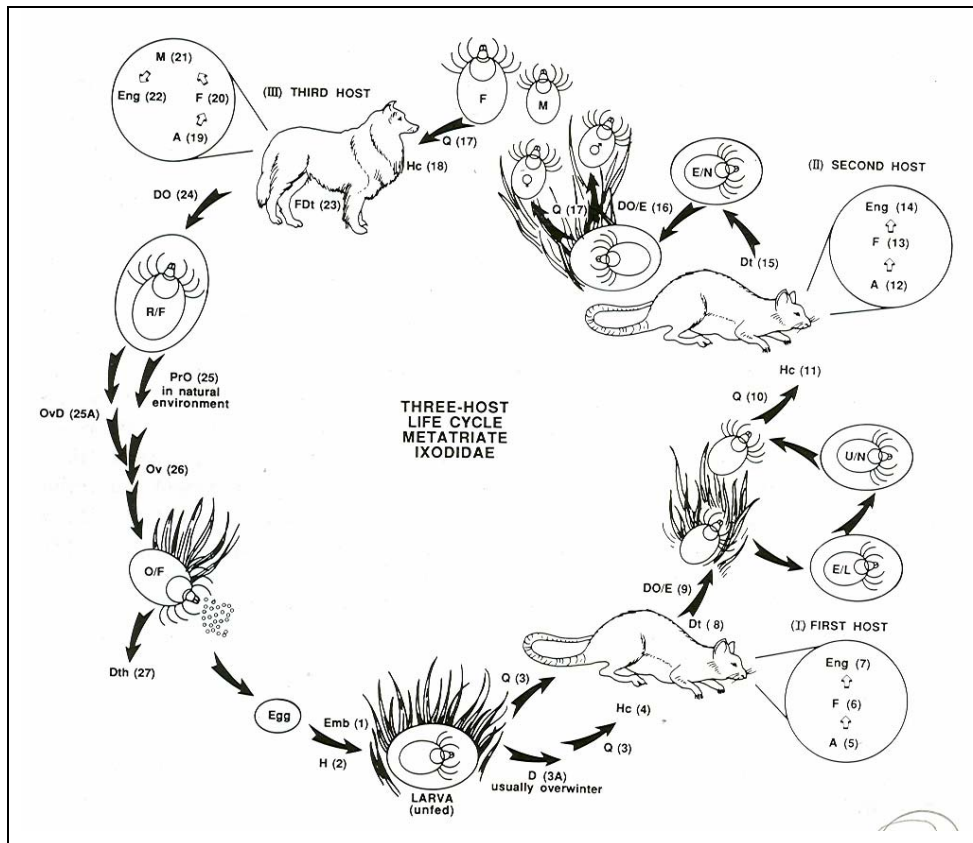


Figure 1.2. Diagram illustrating the typical 3-host cycle characteristic of most ixodid ticks (Sonenshine 1991). The parasitic phases, i.e. when the different life stages are on the host, are shown in the exploded circles. The different events in the life cycle are indicated by a number in parentheses, (1) egg, (2) hatching, (3) larvae, (4) host contact, (5) attachment, (6) feeding, (7) engorgement, (8) detachment, (9) drop off, (10) nymph, (11-15) entire cycle of host contact, attachment, feeding, engorgement, and detachment, (16) nymphs drop off, (17) adults, (18) host contact, (19,20) attachment and feeding, (21) mating, (22) mated females, (23) fed females detach, (24) drop off, (25) pre-ovipositional development, (26) oviposition and (27) death of female.

Argasidae

The family Argasidae comprises 5 genera and approximately 170 species (Sonenshine 1991). In contrast to the Ixodidae, the Argasidae lack the scutum and have a leathery cuticle (Figure 1.3).

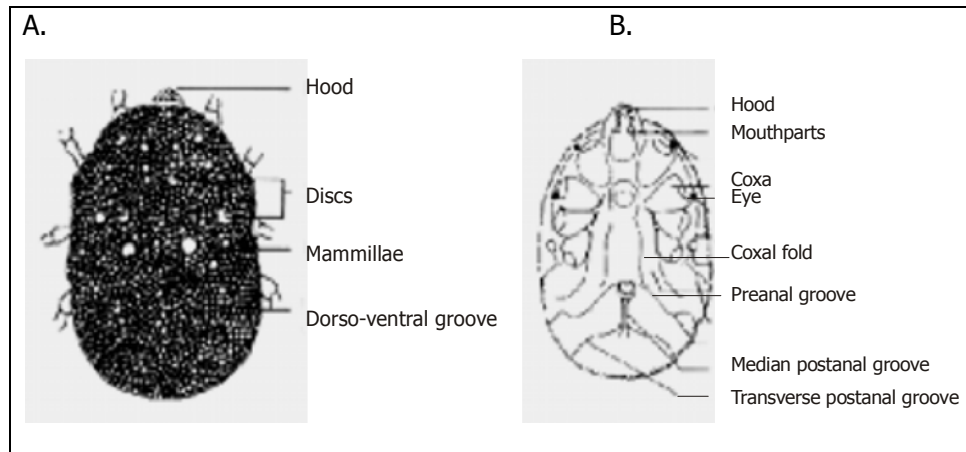


Figure 1.3. Diagram illustrating argasid adult tick body structures (Sonenshine 1991). *The generalized argasid tick ventral view (A) and dorsal view (B) is shown.*

Biologically, argasid ticks differ from ixodid ones in the number of nymphal stages (Figure 1.4). Argasid species may have from 2 to 8 nymphal instars, but usually have only 3 or 4. The number of nymphal instars is not fixed and depends on the size of the blood meal in preceding stages. During feeding, nymphs can increase their body weight 3-5 times, swelling as they feed. Usually males require fewer instars than females (Sonenshine 1991). Argasid ticks exhibit remarkable diversity in their patterns of development and feeding. They feed rapidly and the females oviposit frequently. Similar to mosquitoes and some blood-feeding insects, argasid ticks have multiple gonotrophic cycles (Figure 1.4).

A second characteristic of argasid ticks is the short time in which they feed. Adults and nymphs of most species feed rapidly (minutes to hours), in contrast to the lengthy (days), complex feeding process of ixodid ticks. During feeding, numerous bioactive compounds are secreted from the salivary glands into the host bloodstream (via saliva) to ensure continuous feeding (Table 1.1).

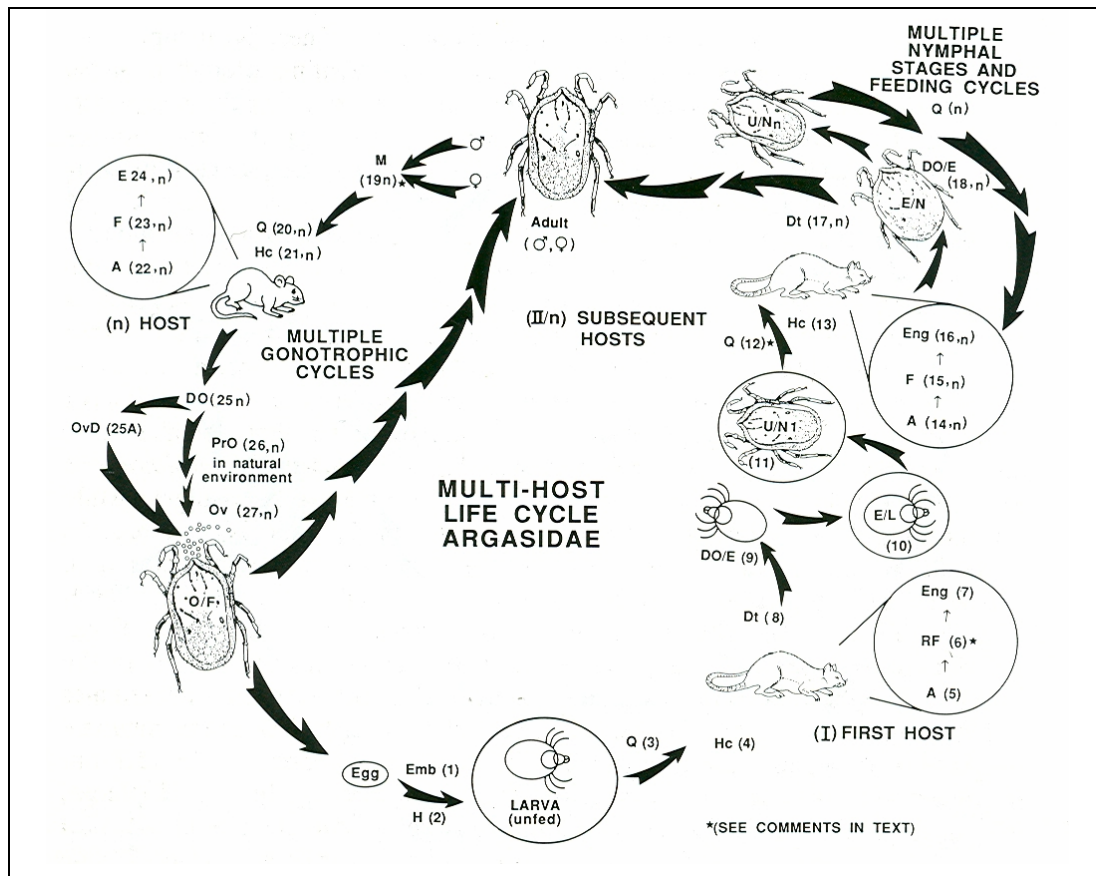


Figure 1.4. Diagram illustrating the typical argasid multi-host life cycle with multiple parasitic phases and repeated gonotrophic cycles (Sonenshine 1991). The different events in the life cycle are indicated by a number in parentheses, (1) embryogenesis, (2) hatching of larvae, (3) host contact, (4) attachment, (5) feeding, (6) engorgement, (7) drop off, (8) ecdyse and molt into first nymphal stage, (9-12 & 13-21) entire cycle of host contact, attachment, feeding, engorgement, and detachment repeat twice, (22) adults mate, (23) host contact, (24) adults feed rapidly, (25) engorge, (26) drop off, (27) mated females oviposit, (28-36) adults seek hosts, feed and engorge several times and fed mated females oviposit after each bloodmeal. The number of gonotrophic cycles is indeterminate.

During this study, the argasid tick *Ornithodoros savignyi* was studied (Figure 1.5). This species is found in sandy regions, giving rise to the local name 'sand tampan', throughout the North Western parts of South Africa and also Egypt, Arabia, Ethiopia, Kenya and Zimbabwe (Paton and Evans 1929). *O. savignyi* is diploid ($2n=20$), with the presence of sex-chromosomes, XY and XX for males and females, respectively (Howell 1966).

Currently these ticks are controlled by chemical treatment of the soil, since ticks avoid these treated areas (Paton and Evans 1929). But since these ticks have a lifespan of 15-20 years and need to feed only occasionally (every 5-6 years), starvation is a not an option (Mans

2002a). Apart from the various bioactive compounds secreted by this species (Table 1.1), much emphasis is placed on it due to its local economic importance in that it kills many domestic animals, especially young calves and lambs. Originally death of the host was contributed to exsanguinations, but later it was shown that death is caused by a toxin which causes serious allergic reactions in humans (Howell *et al.* 1975). Later studies indicated the presence of two toxins (TSGP2 and TSGP4), which affects the cardiac system of the host (Mans *et al.* 2002c).

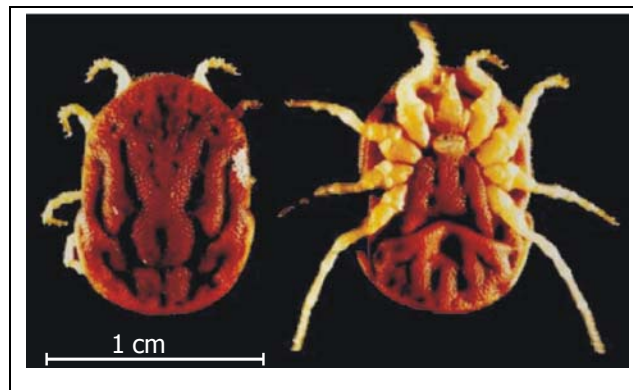


Figure 1.5. External anatomy of a female *O. savignyi*. The folded integument, position of the capitulum and genital opening is visible. Scale bar =1 cm. (Photograph: B.J. Mans, 1996).

Table 1.1. Properties of the granule components secreted by argasid ticks.

Species	Compound	Target in host	Reference
Platelet aggregation factors			
<i>O. moubata</i>	Apyrase	ADP	(Ribeiro <i>et al.</i> 1991)
	Moubatin	Collagen	(Waxman and Connolly 1993)
	TAI	Collagen	(Karczewski <i>et al.</i> 1995)
	Disagregin	$\alpha_{IIb}\beta_3$	(Karczewski <i>et al.</i> 1994)
<i>O. savignyi</i>	Apyrase	ADP	(Mans <i>et al.</i> 1998)
	Savignygrin	$\alpha_{IIb}\beta_3$	(Mans <i>et al.</i> 2002b)
Anticoagulants			
<i>O. moubata</i>	Inhibitors	fxa	(Waxman <i>et al.</i> 1990)
		Trombin	(van de Locht <i>et al.</i> 1996)
<i>O. savignyi</i>	Inhibitors	fxa	(Gaspar <i>et al.</i> 1996; Joubert <i>et al.</i> 1998)
		Trombin	(Nienaber <i>et al.</i> 1999)
Toxins			
<i>Argas (P.) walkerae</i>	Paralysis toxin	Na ⁺ channel	(Viljoen <i>et al.</i> 1990; Maritz <i>et al.</i> 2000; Maritz <i>et al.</i> 2001)

Nuttalliellidae

This family is based on a single species, *Nuttalliella namaqua*, specimens of which were collected from localities in Namibia, South Africa and Tanzania from nests of rock hyraxes and swallows. Nuttalliellidae exhibits features of both the ixodid and argasid ticks. Female have a pseudo-scutum, which is a plate-like structure resembling the scutum in outline. It does not, however, share the smooth appearance of the sclerotized plate of the Ixodidae. Unique to this family is the fact that ball and socket joints articulate the leg segments, a feature not seen in any other species of tick. Other noteworthy features include the lack of eyes, spiracular plates, genital grooves and also dorso-ventral grooves (Sonenshine 1991).

1.2. BIOGENESIS OF SECRETORY GRANULES

As described above, it is clear that all stages and species of ticks require a bloodmeal in order to molt, oviposit and ultimately survive. During these feeding stages, the salivary glands fulfill a core function in ensuring a continuous blood flow from the host, preventing blood clotting in the tick, and counteracting the host's defense mechanisms. To date, bioactive compounds, which enable these processes, have been identified in all tick species. These are synthesized and stored in the salivary glands in diverse granules/vesicles, and are secreted upon stimulation, i.e. regulated exocytosis. The structure and function of argasid salivary glands and the various granules are discussed in great detail in Chapter 2. In this study, we investigated the proteins involved in regulated exocytosis of the salivary glands of *O. savignyi*. Therefore, the mechanisms underlying granule formation, maturation and regulated exocytosis are of significance to this study.

Secretory granules (SGs) can be viewed as alternative organelles capable of accommodating high concentrations of secretory compounds and sensitive to stimulus-induced exocytosis. In most cells SGs appear to represent an entirely new class of organelle, although in some cell types (e.g. hemopoietic cells) they share several properties with lysosomes, such as containing lysosomal markers, are accessible via the endocytic pathway and dense-core formation occurs in multi-vesicular bodies (Burgoyne and Morgan 2003). Furthermore, conventional lysosomes can undergo stimulus-induced fusion with the plasma membrane in a wide range of cells, and therefore it may be that classical secretory granules evolved from a lysosomal progenitor. In contrast to secretory lysosomes found in hemopoietic cells, granules found in endocrine, exocrine and neuronal cells are products of the biosynthetic pathway alone, i.e. they do not resemble lysosomes (Burgoyne and Morgan 2003).

Initial formation of immature granules (ISGs) occurs at the *trans*-Golgi network (TGN) and does not require a coat-driven budding process. Instead it is thought that membrane deformation may result from the aggregation of secretory proteins in the TGN (Burgoyne and Morgan 2003). Once formed, immature SGs must be processed and remodeled to form mature secretory granules (MSGs). In endocrine and neuroendocrine cells this involves both fusion with other immature secretory granules and removal of misrouted material via budding to increased size and density of mature granules (Burgoyne and Morgan 2003). Proteins retained in the MSG are called regulated secretory proteins (RSPs) and secretion of these will only occur from MSGs when the cell receives an external stimulus (Tooze *et al.* 2001). The widely accepted model for the biogenesis of secretory granules comprises four distinct steps (Figure 1.6). These steps are: (1) aggregation of the RSP and sorting of the RSP to the membrane in the TGN, (2) budding from the TGN, (3) homotypic fusion of ISGs, and (4) remodeling of the ISG membrane and content (Tooze *et al.* 2001).

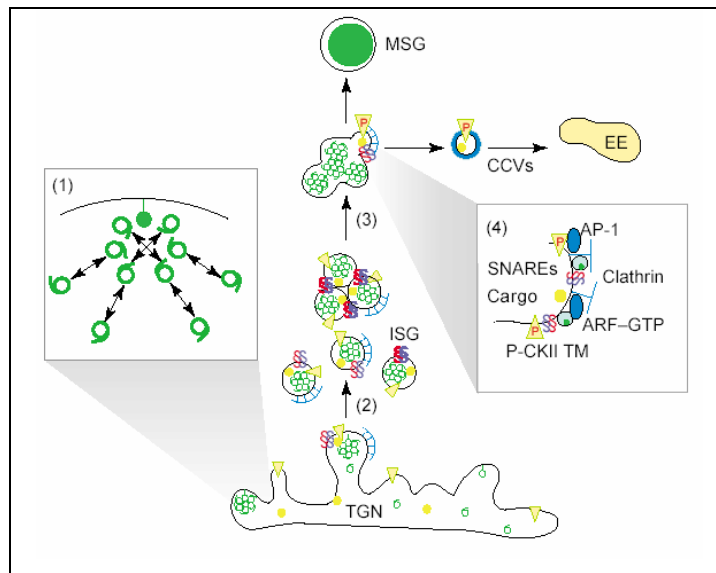


Figure 1.6. Biogenesis of secretory granules in neuroendocrine cells (Tooze *et al.* 2001). Step (1) Regulated secretory proteins (RSPs; green spheres with curly tail) become associated with a specialized region of the trans-Golgi network (TGN) and assemble into oligomers with each other and with a membrane associated form of RSPs (filled green circle with stalk) in the lumen of the TGN. The assembly of the RSPs into oligomers, and later multimers, is the basis of the aggregation of the RSPs. (2) Additional components, including SNARE molecules (blue and red 'S'), other soluble non-secretory granule proteins, or cargo (yellow spheres) and non-secretory granule membrane proteins (yellow triangles), are included into the nascent bud. Immature secretory granules (ISGs) form from the specialized region of the TGN containing the RSP, the cargo and the SNAREs. (3) After formation, ISGs undergo homotypic fusion mediated by SNAREs, followed by membrane remodeling (4). Remodeling is achieved by the interaction of GTP-bound ADP-ribosylation factor (ARF), AP-1 and clathrin to form clathrin-coated vesicles (CCVs), which remove SNAREs, cargo and non-secretory granule membrane proteins that are not destined for the mature secretory granule (MSG) in a process often dependent on phosphorylation (P) by casein kinase II (CKII). Abbreviation: EE, early endosome.

Aggregation of RSPs

To date much effort has been spent on defining a specific domain of the RSPs responsible for sorting into ISGs. Conventional transfection screening was not successful due to the propensity of exogenous and endogenous RSPs to co-aggregate in the slightly acidic, high calcium milieu of the TGN. Proteins such as the family of granins, that includes chromogranin A, -B, secretogranin and 7B2, could be aggregation vehicles for a variety of proteins. Studies, where chromogranin B (CgB) was expressed in cells where endogenous protein synthesis was shut down, indicated that CgB has an N-terminal loop that acted as a signal for sorting into ISGs in the absence of any other RSPs. Deletion of the N-terminal allowed CgB to be sorted through aggregation only in the presence of other RSPs, indicating that the C-terminal may facilitate sorting indirectly (Tooze *et al.* 2001).

A variety of RSPs, containing a chromogranin-like N-terminal loop, is involved in some aspect of RSP sorting. It is believed that the loop either mediates interactions between monomers of RSPs, or by binding directly to a so-called loop receptor. Mutations and deletions in the loop region of various RSPs, as well as their effects, are summarized in Table 1.2.

Table 1.2. Effects of altered loop-regions in various proteins. (Tooze *et al.* 2001).

Cell Type / Protein	Chemical treatment/ Mutation of loop-domain	Effect
PC12 / CgB	DTT disruption	Constitutive secretion of CgB
GH ₄ C1 / CgB	DTT disruption	No effect
CgA	Neutral pH	Loop mediated homo-dimerization
GH ₄ C1 / CgA	Deleted C-terminal domain	Incorrect sorting
PC12 / CgA	Deleted C-terminal domain	No effect
α 1-antitrypsin (AT)	Fused with N-terminal CgB loop domain	AT no longer constitutively secreted, directed into ISGs
α 1-antitrypsin (AT)	Fused with N- and C-terminal CgB loop domains	Sorted more efficiently into ISGs, increased membrane binding of AT

During granule formation RSPs containing dibasic amino acid cleavage sites are processed by pro-hormone convertases, the so-called PC (Prohormone) enzymes. These enzymes are endopeptidases that process prohormones and require low pH and calcium (Tooze *et al.* 2001). Furin is related to the PC enzymes and is responsible for the processing of PCs in the Golgi. Inhibitors of these enzymes include chaperones, serpins and granin-like proteins. The most important inhibitor found in secretory granules is a serpin called endopin 1, which functions by inhibiting basic-residues cleaving proteases (Tooze *et al.* 2001). Since the initiation and efficiency of processing by PC enzymes varies depending on the cell-type and the RSPs, the question as to whether PCs are relevant in sorting is a much debated topic (Tooze *et al.* 2001).

Some RSPs adopt different strategies and rely on different signals to ensure inclusion into ISGs. Examples of the latter include prolactin and growth hormone, which forms a detergent-insoluble aggregate after it exits from the endoplasmic reticulum and, unlike other RSPs such as CgB, does not require an acidic pH to aggregate (Tooze *et al.* 2001). Another strategy that RSPs might use to promote inclusion into ISGs is the recruitment of

aggregation chaperones. The first chaperone to be found in the lumen of the secretory pathway was the granin 7B2 protein (Tooze *et al.* 2001). This protein binds to proPC2 and is essential for its transport from the ER to the ISG. Studies on the expression of his-tagged 7B2 *in vivo* indicated an increase in the aggregation of pro-enkephalin and CgA. The question must, however, be asked whether the overexpression of his-tagged proteins influence the concentration of divalent cations or pH in the TGN, and hence creating a more favorable environment for the RSP to aggregate.

Sorting of RSPs

Association of RSPs with the membrane is required for efficient sorting into nascent ISGs. Although none of the well-studied RSPs are membrane proteins, evidence suggests that many RSPs exist in two forms: a soluble form and a form that is tightly associated with the membrane. From this data several models have been proposed to explain the association of RSPs with the TGN to form ISGs, i.e (1) a subpopulation of RSPs that are tightly associated with the membrane, (2) a membrane protein or (3) a lipid. To date, a combination of models 1 and 3 are favored and is depicted in Figure 1.7.

It is believed that clustering of the soluble and membrane-bound forms of RSPs results in the formation of large aggregates in a specialized subdomain (lipid raft) of the TGN from which an ISG will form. This has been proven for epithelial cells as well as thyroglobulin (a RSP of the thyroid) where the RSP is targeted to a lipid microdomain. By manipulating the lipid levels in cells, the role of cholesterol and sphingolipids could be demonstrated (see Table 1.3).

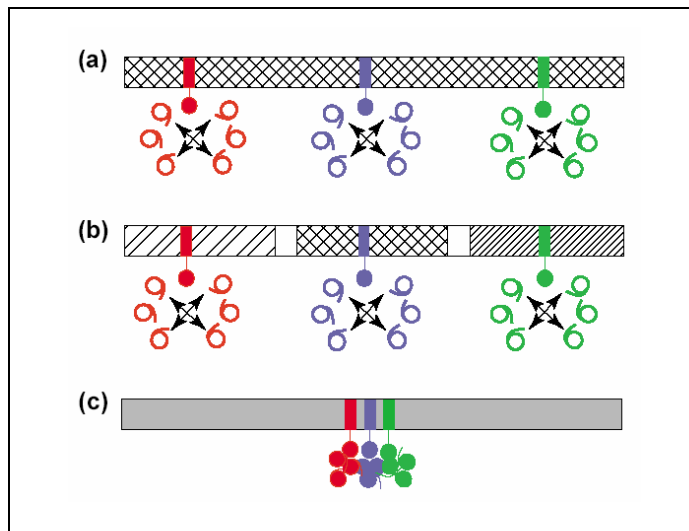


Figure 1.7. Sorting of regulated secretory proteins (RSPs) in the *trans*-Golgi network (TGN) by protein–lipid interactions (Tooze *et al.* 2001). A subpopulation of RSPs is membrane-associated owing to their interaction with lipids. These protein/lipid complexes are contained within lipid rafts (hatched or striped boxes). The different membrane associated RSPs (filled circles shown in red, blue and green) bind to a lipid (rectangles shown in red, blue and green) either in (a) a single lipid raft or (b) separate lipid rafts. Aggregation of the different RSPs with each other, mediated by the N-terminal loops and aggregation domains, and perhaps the associated lipids, promotes the assembly of a specialized domain in the TGN (gray shading) from where immature secretory granules (ISGs) will form (c).

Table 1.3. Examples of RSPs associated with lipid microdomains (Tooze *et al.* 2001).

Cell Type	Chemical treatment	Effect	Reference
AtT20	Lovostatin (Depletes cholesterol)	- Inhibition of secretory granule formation - Reversible upon addition of cholesterol	(Wang <i>et al.</i> 2000)
Various	Addition of cholesterol	Increased ISG production	(Wang <i>et al.</i> 2000)
Chromaffin	None	PC2 and GPIII are associated with lipid rafts	(Palmer and Christie 1992; Blazquez <i>et al.</i> 2000)
AtT20	Fumonisin (Inhibitor of sphingolipid biosynthesis)	Sphingolipids are required for PC2 sorting	(Blazquez <i>et al.</i> 2000)
Bovine pituitary	Methyl- β -cyclodextrin (Deplete cholesterol)	- CPE no longer sorted and occur in soluble fraction - Reduction in the N-terminal loop of POMC binding to granule membranes	(Dhanvantari and Loh 2000)

Maturation of ISGs

Surprisingly, in some cell types immature granules can undergo regulated exocytosis, indicating that the process of granule maturation does not confer plasma membrane fusion competence, but rather inhibits homotypic granule-granule fusion. In contrast, maturation actually increases stimulus-secretion coupling in other cells, as newly formed granules are poorly responsive to secretagogues relative to mature granules (Burgoyne and Morgan 2003).

(a) Remodeling of ISGs

In all endocrine and neuroendocrine cells studied to date, a feature of maturation is the removal of soluble proteins, peptides and membrane proteins from the ISG (Step 3, Figure 1.6). Soluble peptides and hormones removed from the ISG often appear to be secreted from the cell in a non-regulated manner, leading to the description of 'constitutive-like' secretion. Removal of membrane proteins occurs in clathrin-coated vesicles (CCVs), which are targeted to either endosomes or the TGN (Tooze *et al.* 2001). The CCVs may also function to remove soluble proteins destined for constitutive-like secretion.

In AtT20 cells newly formed granules were found to be poorly responsive to secretagogues relative to mature granules. This is believed to be due to the presence of synaptotagmin IV on immature granules that inhibits the putative exocytotic calcium receptor synaptotagmin I. Once it is removed during maturation, normal calcium-dependent release occurs (Burgoyne and Morgan 2003).

(b) Homotypic fusion

Maturation of ISGs involves a change in size; either increased via homotypic fusion or decreased via budding of vesicles from the ISG (Step 3, Figure 1.6). Reconstitution of homotypic fusion in cell-free extracts reveals that the cytosolic components NSF and α -SNAP (involved in all membrane fusion events) are required for ISG-ISG fusion by priming the SNAREs (SNAP receptors). Since the SNAREs present on ISGs differ from those of MSGs, it is hypothesized that they are essential for maturation. To date the mechanism underlying maturation of ISGs by SNAREs has been proven for VAMP 4 (Eaton *et al.* 2000) and syntaxin 6 (Klumperman *et al.* 1998; Dittie *et al.* 1999), which is believed to act by recruiting the proteins ARF and AP-1 (Figure 1.6).

1.3. THE EXOCYTOTIC PATHWAYS

Regulated exocytosis has been dissected into a number of functionally defined, sequential stages (Burgoyne and Morgan 2003). These include physical movement of vesicles to the subplasmalemmal region of the cell, tethering and then docking at release sites on the plasma membrane, ATP-dependent priming steps to convert the vesicles into a fully releasable state, triggered membrane fusion, release of granule content, and finally retrieval of the granule membrane (Figure 1.8)

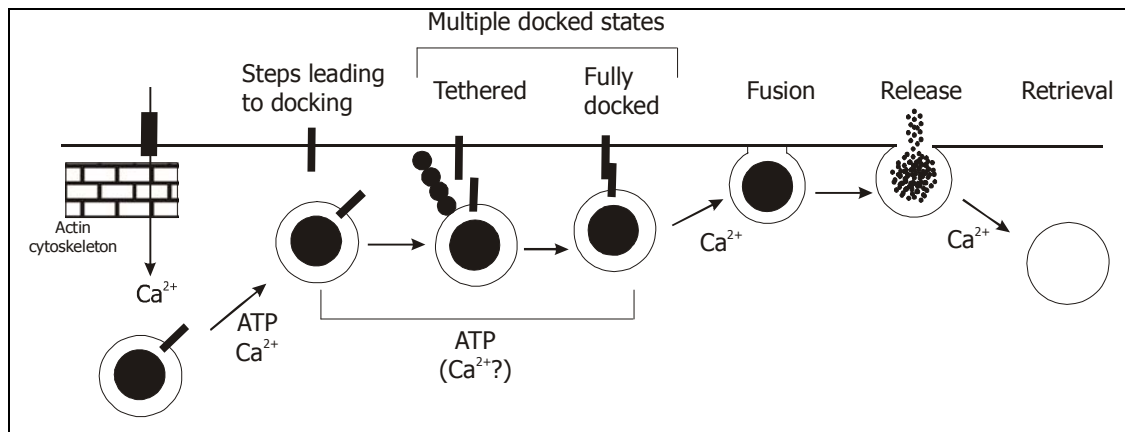


Figure 1.8. Schematic representation of the steps leading to secretory granule exocytosis (Burgoyne and Morgan 2003). This representation is based on neuroendocrine cells undergoing calcium-triggered exocytosis. In this case, calcium entry leads to the disassembly of the cortical actin cytoskeleton, allowing granule recruitment to the plasma membrane where tethering and docking of the granule can occur, followed by fusion and release of granule contents.

Tethering and Docking

The first physical linkage between two membranes destined to fuse appears to be mediated by tethering factors, which may also trigger the engagement of *trans*-SNARE complexes (Ungar and Hughson 2003). Although tethering factors are structurally diverse, they can be divided into two main classes (Table 1.4). Firstly, a group of long coiled-coil proteins that form homodimeric coiled coils with lengths up to several times the diameter of a vesicle. The current model suggests that they are anchored at one end to a membrane, which allows them to search the surrounding space for a passing vesicle, which is then bound by the other end (Whyte and Munro 2002). Secondly, a group of large, hetero-oligomeric complexes have been proposed to play a role in vesicle tethering at distinct trafficking steps. To date, seven conserved complexes have been identified and described (Table 1.4). These tethering complexes are also known to interact with various SNAREs and Rab GTPases located

throughout the secretory pathway (Ungar and Hughson 2003). The various interacting partners are listed in Table 1.4.

Table 1.4. Properties and binding partners of tethering proteins (Lipschutz and Mostov 2002; Whyte and Munro 2002; Ungar and Hughson 2003). Abbreviations of subcellular compartments: (TGN) trans-Golgi network, (PM) plasma membrane, (ER) endoplasmic reticulum.

Tethering Protein	Location <i>in vivo</i>	Receptor	Interacting SNARE / Rab
Coiled-coil proteins			
p115/Uso1p	Golgi	On vesicle: Giantin On Golgi: GM130	Bet1, Bos1, Sec22, Ykt6, Gos28, Membrin, syntaxin 5
EEA1	Early endosome	Unknown	Syntaxin 6 & 13
Hetero-oligomeric complexes			
COG (Conserved oligomeric Golgi complex)	Golgi	Unknown	Bet1, Sec22, Ykt6, Gos1p, Sed5p,
GARP / VFT (Golgi-associated retrograde protein)	Golgi	Unknown	Tlg1p
TRAPP I and II (Transport protein particle)	TGN, Cytosol	Unknown	Bet1
Exocyst (Sec6/8 complex)	PM, TGN	Unknown	Sec4, Rho3, RalA, Rho1
HOPS/ C-Vps complex	Vacuole	Unknown	Vam3
Dsl1p complex	Golgi / ER	Unknown	Sec22, Ufe1

After tethering of the granule/vesicle, *trans*-SNARE complexes are formed. 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, and the SNARE-hypothesis was formed. The latter states that each class of transport vesicle contains a specific protein (v-SNARE) that is capable of associating with a receptor protein (t-SNARE), specific to the appropriate acceptor membrane (Sollner *et al.* 1993; Bajjalieh and Scheller 1995). Later studies, however, indicated that the specificity is not due to SNARE-interactions alone, but is the product of numerous interactions involving non-SNARE proteins. Since the

SNARE proteins are the ones investigated during this study, they are discussed in more detail in the following chapters.

Priming

In neuronal and non-neuronal systems there is substantial evidence that docked vesicles are not intrinsically fusion-competent (Klenchin and Martin 2000). Priming is an essential pre-fusion step, determining the release probabilities of a vesicle by rendering them competent for Ca^{2+} -triggered fusion (Figure 1.9). This has been well described for small synaptic vesicles (SSVs), but currently it is believed that priming of SSVs differ from that of large dense core vesicles (LDCVs) in being more efficient. Rapidly releasable pool (RRP) sizes for LDCVs are estimated as ~ 100 vesicles out of a membrane-docked pool of ~ 1000 LDCVs in chromaffin cells, in contrast to neurons where the releasable pool corresponds closely to the full docked complement of SSVs (Martin 2003). In studies of secretory granule exocytosis, priming is a term used to describe any functionally detected ATP-dependent process that occurs before fusion (Burgoyne and Morgan 2003).

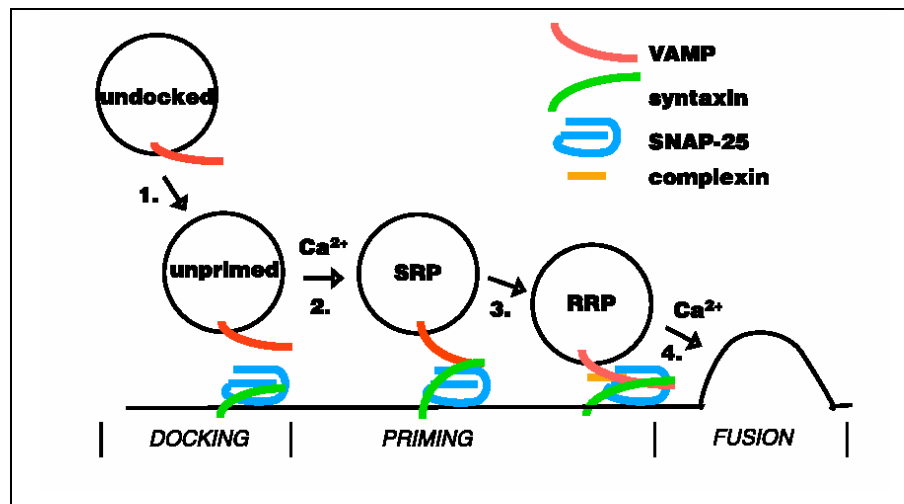


Figure 1.9. LDCV exocytosis viewed as sequential stages of docking, priming and fusion (Martin 2003). Cytoplasmic or undocked vesicles undergo docking (1). Priming of docked vesicles may occur in two stages to generate SRP (slowly releasable pool) and RRP (rapidly releasable pool) of vesicles (2 and 3). The first priming step requires moderate increases in cytoplasmic Ca^{2+} . Activating levels of Ca^{2+} trigger fusion of vesicles from either the SRP (not shown) or from the RRP (4). Each step is thought to be reversible although they are depicted as single arrows in the figure. Priming reactions are thought to involve assembly of trans SNARE complexes, which may be initiated by anchoring of N-terminal domains of SNARE motifs and their subsequent "zippering" to C-terminal, membrane proximal regions. In SV exocytosis, complexins may stabilize trans SNARE complexes at a late stage of assembly rendering them fusion-ready upon Ca^{2+} influx.

To date, p145/CAPS has been identified as essential for the priming of LDCVs by rendering the LDCVs calcium-sensitive (Martin 2003). Furthermore, CAPS is also regarded as a tethering protein since it interacts with both the LDCV and the plasma membrane via PIP₂ (Phosphatidyl inositol 4,5-bisphosphate; which is essential for LDCV exocytosis). In PC12 cells, the priming reaction for which CAPS is essential is strongly ATP-dependent. It is believed that ATP is required for maintaining the slow releasable vs. the rapid releasable pool of vesicles, thereby enabling its replenishment (Martin 2003).

Synaptotagmins (Syt) are a second class of protein involved in priming of LDCVs. In PC12 cells cross-linking studies showed that Syt proximity to SNARE complexes increases when exocytosis-triggering levels of calcium are provided. This suggests that the majority of docked LDCVs in PC12 cells are in an unprimed state in which *trans*-SNARE complex formation has yet to be initiated. The calcium dependence of the priming step may be mediated by Syt proteins, which catalyze *trans*-SNARE complex formation (Martin 2003).

The ATPase NSF (N-ethylmaleimide sensitive factor) has also been linked to priming of vesicles in non-neuronal cells, although not conclusively to dense core vesicles. In PC12 cells, neutralizing NSF antibodies were found to inhibit the ATP-dependent priming of docked vesicles, indicating that NSF may function to promote the ATP-dependent disassembly of *cis*-SNARE complexes that are largely resident on the plasma membrane of PC12 cells (Klenchin and Martin 2000). In chromaffin cells, N-ethylmaleimide treatment resulted in a reduced refilling of the rapidly releasable pool (RRP) of vesicles, similar to the results obtained in a temperature-sensitive allele of *Drosophila* NSF. Further studies indicated that docked vesicles accumulated at the membrane, but that they were fusion-incompetent, indicating that NSF functions in vesicle priming (Klenchin and Martin 2000). Finally, the protein Munc13-1 is also believed to act as a priming factor, since overexpression promotes priming of LDCVs in chromaffin cells (Martin 2003).

Interesting to note is the fact that priming is regulated by various protein kinases. In chromaffin cells, phorbol ester stimulation of protein kinase C (PKC) increased the size of the RRP and enhanced the rate of refilling the RRP without affecting vesicle fusion probabilities, an indication that PKC regulates vesicle priming. The role of protein kinase A (PKA) during priming has been shown in β -insulin secreting cells (Klenchin and Martin 2000).

Membrane fusion

In 1980 Chandler and Heuser reported the first image of an expanding fusion pore in degranulating mast cells by using rapid freezing techniques. They were able to show that membrane-lined pores of 20-100 nm in diameter provided a water path for secretory products to exit the vesicle interior into the extracellular space. Due to the smoothness of the membrane lining the pores, it was believed that pores are made exclusively of lipids (Lindau and deToledo 2003). By using more advanced techniques, such as admittance analysis (which monitor changes in cell surface areas), the groups of Almers and Zimmerberg independently provided the first estimations of fusion pore conductance and kinetics in degranulating mouse mast cells (Lindau and deToledo 2003). It was concluded that the initial fusion pore conductance was 200-300 pS (pico Siemens) in a lipid bilayer with a diameter of ~ 2 nm. This value, similar to the conductance of the potassium channel or a gap junction channel, is consistent with a fusion pore made of proteins. Later experiments by Monck and Fernandez indicated fusion pore conductances as low as 50 pS in eosinophils and neutrophils, which proposed that fusion pores are lipidic. These two extreme hypotheses led to a debate still unresolved (Lindau and deToledo 2003).

Another debate regarding membrane fusion entails full fusion versus transient fusion. Full fusion means that following the formation of a fusion pore, the pore expands to a large size and the vesicle membrane becomes fully incorporated into the plasma membrane. Transient fusion (kiss-and-run fusion) means that the pore opens, and maybe expands, but then closes again in order for the vesicle to retain its integrity when it discharges its contents (Figure 1.10)

Although to date no clear model exists for the fusion pore, it is widely believed that pore formation is a consequence of structural changes in the SNARE complex. Present models envision the C-terminal ends of VAMP and syntaxin to be located close to each other in the center of the pore, while the C-terminal of SNAP25 is located near the transmembrane domains of VAMP and syntaxin (Lindau and deToledo 2003). The SNARE hypothesis of fusion suggests that the initial fusion pore may be a proteolipid structure. In neurons and chromaffin cells, the SNARE proteins VAMP, syntaxin and SNAP25 are thought to form and expand the fusion pore. The dependence of membrane fusion on the concentration of soluble VAMP2 coil domains has suggested that three SNARE complexes mediate fusion of a vesicle (Lindau and deToledo 2003).

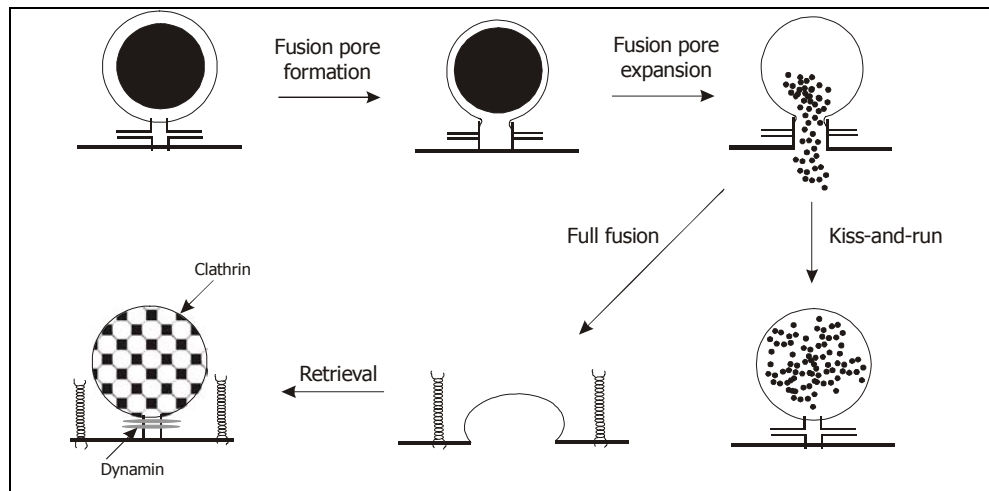


Figure 1.10. Comparison of kiss-and-run exocytosis and full fusion (Burgoyne and Morgan 2003). Membrane fusion initially occurs by the formation of a fusion pore. Fusion pore expansion allows release of granule content. This can be limited by rapid re-closure of the fusion pore (kiss-and-run). Alternatively, full emptying of the granule can be followed by a retrieval mechanism involving dynamin and clathrin.

The SNARE fusion hypothesis is, however, not accepted by all scientists, since SNAREs could only be responsible for membrane merging with fusion being executed by other proteins (Jahn and Grubmuller 2002). Support for the latter came from (a) the identification of several proteins, which operate downstream of SNAREs, such as protein phosphatase I, calmodulin, the V_0 subunit of the vacuolar ATPase and Vac8 as well as (b) observations showing that SNAREs appear to be expendable for fusion (Jahn and Grubmuller 2002).

Fusion pores also seem to be influenced by calcium levels. In PC12 cells, overexpression of syt I prolonged the lifetime of the fusion pore, whereas syt IV shortened this time. Later studies performed in eosinophils indicated that initial fusion pore formation does not depend on the intracellular calcium concentration, but that the rate of fusion pore expansion is regulated by the intracellular calcium concentration. A role for activation of PKC was also shown to influence pore expansion (Lindau and deToledo 2003).

In biological systems, the tight coupling between exocytosis and endocytosis must always be considered. Rapid endocytosis is often considered equivalent to kiss-and-run, although direct evidence is usually not available. This was shown in PC12 cells where membrane sheets revealed that ~30% of exocytosed vesicles were recaptured. The recapturing was not simple fusion pore flickering, but involved dynamin, indicating a tight coupling between exo- and endocytosis (Lindau and deToledo 2003).

1.4. PROTEIN-PROTEIN INTERACTIONS: A TARGET FOR THERAPY?

Protein trafficking and secretion, similar to various other cell processes, occur via protein-protein interactions. Proteins interact in complicated ways because of the complexity of their 3-dimensional structures. Amino acid side chains that are exposed on the surface of the molecule create pits or bumps of different shapes and sizes, which are then exploited by proteins producing binding pockets and recognition sites with varying degrees of specificity and subtlety of interaction. It is this versatility of protein-protein interactions that makes them such a tempting prospect to exploit in the search for new drugs (Buckingham 2004).

Apart from being promising, targeting protein-protein interactions present serious obstacles such as the range of concentrations over which proteins might interact. Affinities between proteins can vary depending on the cellular function and the immediate chemical environment, especially the pH and calcium concentration. Peptides and small molecules used for disrupting protein interactions must also be able to reach the inside of the cell as well as the site of interaction.

To date, two methods have proven successful in identifying new targets for disrupting protein-protein interactions. Dale Boger at the Scripps Research Institute, San Diego, was the first to be successful in using peptidomimetics, i.e. short, synthesized peptide fragments that mimic the most common motifs such as α -helix or β -sheet (Buckingham 2004). He used a technique called solution-phase combinatorial chemistry to generate a library of some 40,000 variants of these peptidomimetics and was successful in identifying a compound that blocks the interactions between integrin ($\alpha_v\beta_3$) and MMP2, two proteins that initiate angiogenesis in tumors. This compound was found to interfere with the site that controls the localization of MMP2 within the cell (Buckingham 2004).

An alternative approach to combinatorial chemistry is to screen a number of small organic compounds, called fragments, in order to identify the ones that bind to the protein of interest (Buckingham 2004). These small molecules can either bind directly to the site of interaction, or can allosterically induce conformational changes that hides the desired domain (Dev 2004). The fragments can be screened using nuclear magnetic resonance, or by an ingenious technique called 'tethering' which was pioneered by Jim Wells from Sunesis Pharmaceuticals (Buckingham 2004). Tethering commences by engineering the target protein with a cysteine mutation near a known interaction site. The protein is then probed

with test fragments containing disulphide bonds. When a fragment hits the target area, it becomes chemically bonded to the cysteine and can subsequently be identified using mass spectroscopy. The main advantage of this approach is that it starts with an enriched source of lead compounds, themselves good starting points because they are less hydrophobic and are therefore likely to be good building blocks for drugs. To date the group of Wells was successful in identifying two sub-sites on the interleukin receptor, IL-2, a rigid one and a more flexible one, of which the latter proved more effective in binding the fragment (Buckingham 2004).

SNARE proteins, which are involved in exocytosis, have been extensively investigated for the identification of new therapeutics because of the implication of neuro-secretion in the pathogenesis of several human neurological disorders (Blanes-Mira *et al.* 2003). Thus far, clostridial neurotoxins and peptides patterned after the protein domains of SNAREs have been the only molecules able to modulate the assembly and stability of the core complex (Apland *et al.* 2003; Blanes-Mira *et al.* 2003). One such an example is that of small peptides patterned after the N-terminal domain of SNAP25 (Figures 1.11 and 1.12). Two peptides, which are identical to the N-terminal of native SNAP25, SNAP25_N1 (residues 1-21) and SNAP25_N2 (residues 22-44), inhibited 25% and 50% of complex formation, respectively. Mechanistically, the peptides act by disrupting the interaction of the parental protein (SNAP25) with syntaxin (Figure 1.11). By using another peptide SNAP_N4, which resemble the core of peptide SNAP25_N2 (residues 26-28) it was shown that the SNAP25_N4 peptide can establish up to seven interactions with syntaxin and the SNAP25 C-terminal. As shown in Figure 1.11, residue 30Arg on the peptide forms a salt bridge with 145Glu on the C-terminal of SNAP25, while 31Arg and 28Ser interact with 206Glu on syntaxin. Furthermore, 36Glu on SNAP25_N4 pairs with 213His on syntaxin. In addition of these interacting pairs, several hydrophobic interactions notably contribute to peptide binding (Inset, Figure 1.11). *In vivo*, these were found to inhibit SNARE complex assembly, regulate inhibited calcium-dependent exocytosis from excitable cells and are able to translocate through the plasma membrane of intact cells (Blanes-Mira *et al.* 2004).

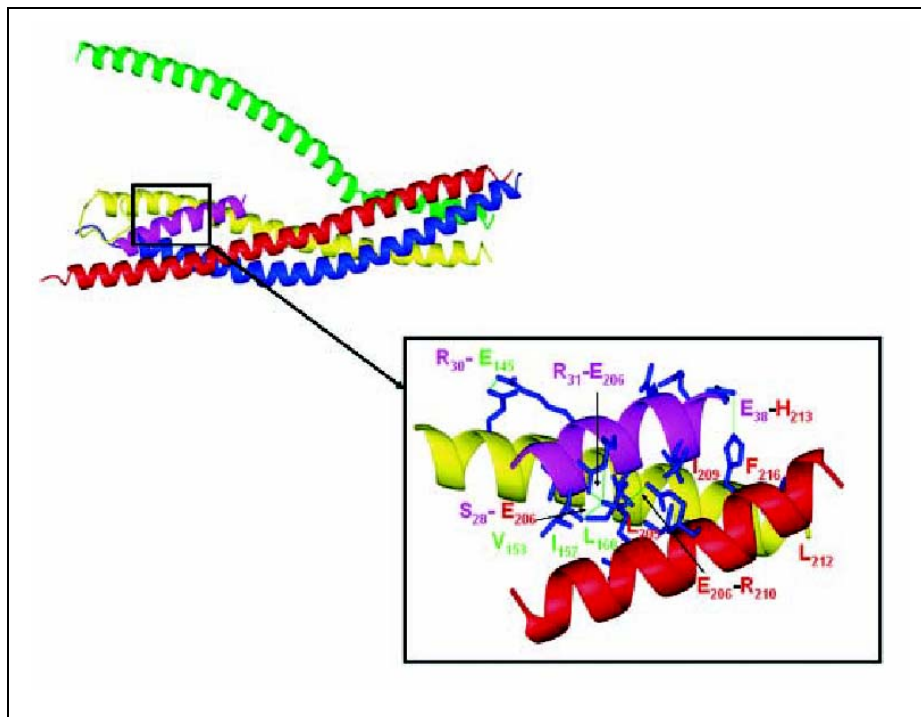


Figure 1.11. Structural model illustrating the putative binding site of peptides SNAP25_N2 on the SNARE complex (Blanes-Mira *et al.* 2004). (Top) Putative interaction of SNAP25_N2 peptide (residues 22-44) on the SNARE complex preventing the interaction of the N-terminus domain of SNAP25. (Bottom) Enlargement showing the interactions of peptide SNAP25_N4 (residues 26-38) with syntaxin (red), the C-terminus of SNAP25 (yellow) and VAMP (blue). Peptide is shown in magenta. Interactions are highlighted.

The group of Ferrer-Montiel has recently provided the first description of peptides with sequences unrelated to the SNARE proteins that are capable of inhibiting the assembly of the core complex. They were able to identify SNARE modulators that inhibit exocytosis from an α -helix constrained, mixture-based, 17-mer combinatorial peptide library composed of 137 180 sequences (Blanes-Mira *et al.* 2003). By screening the peptides for their ability to prevent the formation of the SDS-resistant SNARE core complex, eight peptides were identified. The most potent 17-mer peptide (acetyl-SAAEAFKLYAEAFKGNH₂, Figure 1.12.A) abolished both calcium-evoked catecholamine secretion from chromaffin cells and L-glutamate release from hippocampal primary cultures (syntaxin, SNAP25, VAMP). The structures are shown in Figure 1.12.

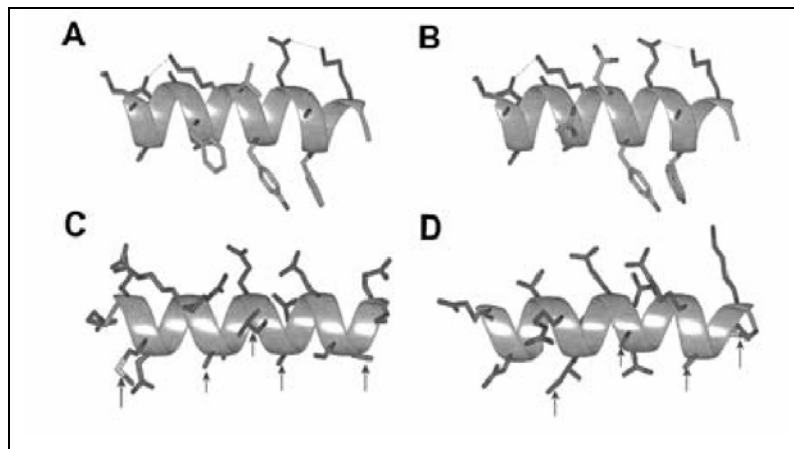


Figure 1.12. α -Helical models of peptides identified from an α -helical constrained combinatorial peptide library (Blanes-Mira *et al.* 2003). Models are shown for the most (SAAEAFAKLYAEAFAG; A) and least SAAEAEAKQYAEAWAKG; (B) active peptides for inhibition of SNARE complex formation, and for peptides patterned after the N-terminus (EEMQRRADQLADESLES; C) and C-terminus (DSNKTRIDEANQRATKM; D) of SNAP25. The helical structure for SNAP25 peptides was obtained from the three-dimensional structure of SNAP25 in the SNARE complex. Arrows indicate hydrophobic residues involved in core coiled-coil interactions in the SNARE complex.

Another novel set of fusion peptides, which inhibits activity of the NSF ATPase and blocks exocytosis, has been described by Matsushita *et al.* (Matsushita *et al.* 2005). These NSF inhibitors are fusion polypeptides composed of an 11 amino acid HIV TAT domain fused to a 22 amino acid NSF domain. These TAT-NSF fusion polypeptides cross endothelial cell membranes, inhibit NSF hydrolysis of ATP, decrease NSF disassembly of the SNARE complex, and block exocytosis (Matsushita *et al.* 2005).

Excitingly, known drugs are being re-investigated to determine their mode of action. In some cases, they are only now being linked to SNARE proteins. One such an example is the anti-epileptic drugs carbamazepine (CBX) and zonisamide (ZNS). Recent findings indicate that in neuronal cells they reduce P-type voltage-gated calcium channel/synaptobrevin-related exocytosis mechanisms during the depolarization stages, and simultaneously enhance N-type voltage-gated calcium channel/syntaxin-related exocytosis mechanisms at the resting stage (Okada *et al.* 2002).

Taking into account the advances in this field during the past few years, targeting protein-protein interactions in order to inhibit exocytosis, seems feasible. If one can identify and study the proteins and their interacting partners regulating exocytosis in the salivary glands of ticks, it would be possible to design or screen for an anti-tick feeding compound.

1.5. AIMS OF THIS THESIS

By understanding the mechanism of exocytosis in tick salivary glands, the various pathways and compounds regulating the process can be used for the rational design of an anti-tick feeding drug or vaccine. In the long run, successful disruption of exocytosis can inhibit tick feeding (hence the lifecycle of ticks) and possibly also affect pathogen transmission.

During this study we investigated the signaling pathways, as well as the proteins involved in regulated exocytosis of protein from large dense core granules from the salivary glands of *O. savignyi*. Since significant information is available regarding the signaling pathways of ixodid ticks, we compared the mechanism to that found in the argasid tick, *O. savignyi*.

Furthermore, since SNAREs and their binding partners have been implicated in controlling regulated exocytosis, we used molecular biological tools as well as methods exploiting protein-protein interactions, in order to identify homologous partners in *O. savignyi*. Antibodies (Chapter 3), degenerative primers (Chapter 3), the yeast two-hybrid system (Chapter 4), functional complementation in yeast (Chapter 5) and affinity chromatography with recombinant SNAREs (Chapter 5) were used.

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