

**The mechanisms regulating exocytosis of the salivary glands  
of the soft tick, *Ornithodoros savignyi***

by

Christine Maritz-Olivier

Submitted in partial fulfilment of the requirements for the degree

*Philosophiae Doctor*

in the

Faculty of Natural and Agricultural Science

Department of Biochemistry

University of Pretoria

Pretoria

July 2005

## CONTENTS

List of Abbreviations .....	vi
List of Figures .....	xi
List of Tables .....	xix
Acknowledgements .....	xxii

### **Chapter 1: Literature review**

1.1. Ticks: An overview .....	1
1.2. Biogenesis of secretory granules .....	6
1.3. The exocytotic pathways .....	13
1.4. Protein-protein interactions: A target for therapy?.....	19
1.5. Aims of this thesis.....	23
1.6. References .....	24

### **Chapter 2: Signaling pathways regulating protein secretion from the salivary glands of unfed female *Ornithodoros savignyi*.**

2.1. Introduction.....	27
2.1.1. General anatomy of tick salivary glands .....	27
2.1.2. Extracellular stimuli.....	31
2.1.3. Adenylyl cyclase and cAMP .....	35
2.1.4. Prostanoids .....	38
2.1.5. Phospholipase C and intracellular calcium.....	43
2.1.6. Current model for the control and mechanism of secretion in ixodid ticks .....	44
2.2. Hypothesis.....	46
2.3. Aims .....	46
2.4. Materials .....	47
2.5. Methods .....	47
2.5.1. Tick salivary gland dissection.....	47
2.5.2. Apyrase activity assay .....	47
2.5.3. Agonist and antagonist treatment.....	49
2.5.4. Phosphorylation assay.....	49
2.6. Results and discussion.....	50

2.6.1.	Dopamine / Isoproterenol / Carbachol.....	50
2.6.2.	Intracellular calcium.....	52
2.6.3.	Prostaglandins .....	53
2.6.4.	cAMP .....	55
2.6.5.	Verapamil.....	57
2.6.6.	Ouabain .....	58
2.6.7.	Extracellular and intracellular conditions (Membrane potential).....	60
2.6.8.	N-ethylmaleimide (NEM) .....	61
2.6.9.	GTP $\gamma$ S .....	62
2.6.10.	cAMP-Dependent phosphorylation.....	63
2.6.11.	PI-3-Kinase inhibitor (Wortmannin).....	65
2.6.12.	Inositol (1, 4, 5) tri-phosphate (IP <sub>3</sub> ).....	68
2.6.13.	PLC Inhibitor (U73,122) .....	68
2.6.14.	Actin inhibitor (Cytochalasin D).....	69
2.6.15.	Tubulin Inhibitor (Colchicine).....	71
2.7.	Conclusion.....	73
2.8.	References .....	76

**Chapter 3: Investigations into the conserved core machinery of regulated exocytosis in the salivary glands of *O. savignyi***

3.1.	Introduction.....	80
3.1.1.	Conserved core machinery for regulated exocytosis .....	81
3.2.	Hypothesis.....	98
3.3.	Aims .....	98
3.4.	Materials .....	99
3.5.	Methods .....	99
3.5.1.	Salivary gland fractionation .....	99
3.5.2.	Protein gel electrophoresis .....	100
3.5.3.	Western blotting .....	100
3.5.4.	Immuno-fluorescent localization using confocal microscopy.....	100
3.5.5.	Degenerative primer design.....	101
3.5.6.	Total RNA isolation .....	101
3.5.7.	Conventional cDNA synthesis.....	102
3.5.8.	SUPER SMART™ cDNA synthesis .....	102

3.5.9.	cDNA amplification by LD-PCR .....	104
3.5.10.	Random amplification of 3' cDNA ends (3'-RACE).....	104
3.5.11.	DIG- labelling of probes using PCR .....	105
3.5.12.	DNA dot blotting.....	106
3.5.13.	Agarose gel electrophoresis.....	106
3.5.14.	PCR product purification.....	106
3.5.15.	Quantification of nucleic acids .....	107
3.5.16.	A/T cloning of PCR products into pGEM® T-Easy vector.....	107
3.5.17.	Preparation of electrocompetent cells.....	107
3.5.18.	Transformation by electroporation .....	108
3.5.19.	Miniprep plasmid isolation .....	108
3.5.20.	High pure plasmid isolation .....	108
3.5.21.	Automated DNA sequencing and data analysis.....	109
3.6.	Results and Discussion .....	110
3.6.1.	Western blotting of salivary glands with anti-SNARE and anti-Rab3a antibodies .....	110
3.6.2.	Localization of SNAREs and cytoskeleton proteins using confocal microscopy .....	111
3.6.3.	RNA isolation.....	115
3.6.4.	3'-RACE using ss cDNA.....	116
3.6.5.	3'-RACE using SUPER SMART™ ds cDNA .....	124
3.7.	Conclusion.....	131
3.8.	References .....	133

**Chapter 4: Investigation into protein-protein interactions between rat brain secretory proteins and an *O. savignyi* cDNA library by means of the GAL4 two-hybrid system**

4.1.	Introduction.....	136
4.1.1.	The yeast two hybrid system .....	136
4.1.2.	Using the two-hybrid system for the identification of binding partners of SNAREs and secretory proteins.....	146
4.2.	Hypothesis.....	149
4.3.	Aims .....	149
4.4.	Materials .....	150

4.5.	Methods .....	150
4.5.1.	Full-length GAL4 AD/ library construction .....	150
4.5.2.	Truncated GAL4 AD/ library construction .....	154
4.5.3.	Verification of yeast host strains and control vectors .....	155
4.5.4.	GAL4 DNA-BD/Bait construction.....	156
4.5.5.	Small-scale yeast transformation .....	159
4.5.6.	GAL4 DNA-BD/Bait test for autonomous reporter gene activation.....	160
4.5.7.	Sequential library-scale transformation of AH109 yeast cells.....	160
4.5.8.	Two-hybrid screening of reporter genes .....	161
4.5.9.	Colony-lift $\beta$ -galactosidase filter assay.....	161
4.5.10.	Nested-PCR screening of positive clones .....	162
4.5.11.	Plasmid isolation from yeast .....	163
4.5.12.	AD/library plasmid rescue via transformation in KC8 E. coli .....	163
4.5.13.	Sequencing of AD/library inserts .....	163
4.6.	Results and Discussion .....	165
4.6.1.	Full-length cDNA GAL4 AD / Plasmid library construction.....	165
4.6.2.	Truncated GAL4 AD / Plasmid library construction.....	168
4.6.3.	Bait construction.....	170
4.6.4.	Transformation of bait/ GAL4 BD constructs into AH109.....	175
4.6.5.	Library transformation and two-hybrid screening .....	176
4.6.6.	Colony-lift $\beta$ -galactosidase assay.....	178
4.6.7.	Nested-PCR screening of $\beta$ -galactosidase positive clones.....	178
4.6.8.	Sequencing and analysis of positive AD/library inserts.....	180
4.7.	Conclusion .....	190
4.8	References .....	191

**Chapter 5: Investigating SNARE-interactions by functional complementation in *Saccharomyces cerevisiae* and pull-down assays with  $\alpha$ -SNAP**

5.1.	Introduction.....	194
5.1.1.	<i>S. cerevisiae</i> : A model organism for studying protein transport.....	194
5.1.2.	Functional complementation.....	197
5.1.3.	Functional complementation of SNAREs and trafficking proteins in yeast .....	200

5.1.4.	$\alpha$ -SNAP: Functional properties .....	201
5.2.	Hypothesis.....	204
5.3.	Aims .....	204
5.4.	Materials .....	205
5.5.	Methods .....	205
5.5.1.	<i>O. savignyi</i> salivary gland cDNA library construction.....	205
5.5.2.	Growth and maintenance of SSO-mutated yeast cells.....	206
5.5.3.	Transformation, selection and screening .....	206
5.5.4.	Data analysis.....	206
5.5.5.	Expression of rat brain $\alpha$ -SNAP.....	206
5.5.6.	Salivary gland homogenate preparation .....	207
5.5.7.	Affinity chromatography (Pull-down assays) .....	207
5.5.8.	ELISA.....	207
5.5.9.	SDS-PAGE .....	207
5.6.	Results and Discussion .....	209
5.6.1.	cDNA library construction .....	209
5.6.2.	Growth and maintenance of syntaxin knockout yeast.....	211
5.6.3.	Transformation, selection and screening .....	211
5.6.4.	Data analysis.....	213
5.6.5.	Pull-down assays .....	218
5.7.	Conclusion.....	221
5.8.	References .....	223
<b>Chapter 6: Concluding discussion .....</b>		225
<b>Summary.....</b>		230
<b>Appendix .....</b>		232

## LIST OF ABBREVIATIONS

A	Adenosine / Alanine
AA	Arachidonic acid
AD	Activation domain
Ade	Adenine
AMP	Adenosine monophosphate
Amp	Ampicillin
$\alpha$ SNAP	$\alpha$ -Soluble NSF attachment protein
ATP	Adenosine triphosphate
BD	Binding domain
BLAST	Basic local alignment search tool
bp	Base pairs
$^{\circ}$ C	Degrees Celcius
C	Cytosine / Cysteine
cAMP	Cyclic adenosine monophosphate
CCV	Clathrin-coated vesicle
cDNA	Complementary DNA
cfu	Colony forming units
CgB	Chromogranin B
CHX	Cycloheximide
COX	Cyclooxygenase
C-terminal	Carboxy terminal
D	Aspartic acid
Da	Dalton
dA	Deoxy adenosine
DAG	Diacyl glycerol
dC	Deoxy cytosine
DDO	Double dropout
DEPC	Diethyl pyrocarbonate
dG	Deoxy guanosine

DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNA-BD	DNA-binding domain
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DO	Dropout
ds	Double stranded
dT	Deoxy thymidine
DTT	Dithiothreitol
E	Glutamic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetra acetic acid
EE	Early endosome
EGTA	Ethylene-bis (oxyethylene nitrilo) tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
F	Phenylalanine
G	Guanidine / Glycine
GAL4	Galactose 4 regulatory protein
G <sub>i</sub>	Inhibitory G-protein
G <sub>s</sub>	Stimulatory G-protein
H	Histidine
I	Inosine / Isoleucine
InsP	Insitol phosphate
IP <sub>3</sub>	Insitol 1,4,5-triphosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
ISG	Immature granule
K	Lysine
kDa	Kilo Dalton

L	Leucine
<i>lacZ</i>	$\beta$ -Galactosidase gene
LB	Luria-Berthani
LDCV	Large dense core vesicle
LD-PCR	Long distance PCR
M	Methionine
MCS	Multiple cloning site
$\mu$ M	Micromolar
$\mu$ mol	Micromole
mg	Milligram
min	Minutes
mM	Millimolar
mRNA	Messenger RNA
MSG	Mature secretory granule
N	Asparagine
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NLS	Nuclear localization signal
nmol	Nanomole
NSF	N-Ethylmaleimide sensitive factor
N-terminal	Amino terminal
ORF	Open reading frame
<i>ori</i>	Origin of replication
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Poly-ethylene glycol
PG	Prostaglandin
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>

PIP <sub>2</sub>	Phosphatidyl inositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospho lipase C
pmol	Picomole
pS	picoSiemens
Q	Glutamine
QDO	Quadruple dropout
R	Arginine
RACE	Random amplification of cDNA ends
RNase	Ribonuclease
RNA	Ribonucleic acid
RRP	Rapidly releasable pool
RSP	Regulated secretory protein
RT-PCR	Reverse transcription PCR
S	Serine
SAP	Shrimp alkaline phosphatase
SD	Standard dropout
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SG	Secretory granule
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
SRP	Slowly releasable pool
ss	Single stranded
SSV	Small synaptic vesicle
syt	Synaptotagmin
T	Thymidine / Threonine
TAE	Tris-acetate EDTA buffer
Taq	<i>Thermus aquaticus</i>

TBS	Tris buffered saline
TDO	Triple dropout
TEM	Transmission electron microscopy
TGN	<i>trans</i> -Golgi network
T <sub>m</sub>	Melting temperature
Tris	Tris(hydroxymethyl) aminomethane
tRNA	Transfer RNA
U	Units
UAS	Upstream activating sequences
V	Valine
VAMP	Vesicle associated membrane protein
W	Tryptophan
WT	Wild type
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y	Tyrosine

## LIST OF FIGURES

### Chapter 1:

Figure 1.1.	Diagram illustrating ixodid adult tick body structures.....	1
Figure 1.2.	Diagram illustrating the typical 3-host cycle characteristics of most ixodid ticks.....	2
Figure 1.3.	Diagram illustrating argasid adult tick body structures.....	3
Figure 1.4.	Diagram illustrating the typical argasid multi-host life cycle with multiple parasitic phases and repeated gonotrophic cycles .....	4
Figure 1.5.	External anatomy of a female <i>O. savignyi</i> .....	5
Figure 1.6.	Biogenesis of secretory granules in neuroendocrine cells.....	8
Figure 1.7.	Sorting of regulated secretory proteins (RSPs) in the trans-Golgi network (TGN) by protein–lipid interactions.....	11
Figure 1.8.	Schematic representation of the steps leading to secretory granule exocytosis .....	13
Figure 1.9.	LDCV exocytosis viewed as sequential stages of docking, priming and fusion .....	15
Figure 1.10.	Comparison of kiss-and-run exocytosis and full fusion.....	18
Figure 1.11.	Structural model illustrating the putative binding site of peptides SNAP25_N2 on the SNARE complex .....	21
Figure 1.12.	$\alpha$ -Helical models of peptides identified from an $\alpha$ -helical constrained combinatorial peptide library .....	22

### Chapter 2:

Figure 2.1.	SEM analysis of salivary glands from <i>O. savignyi</i> .....	27
Figure 2.2.	TEM micrographs of the granules of type II granular alveoli .....	31
Figure 2.3.	Biosynthesis of the physiologically active amines dopamine, epinephrine and norepinephrine .....	33
Figure 2.4.	A model to demonstrate the receptors involved in salivary fluid secretion in ixodid ticks .....	35
Figure 2.5.	The mechanism of receptor-mediated activation / inhibition of adenylyl cyclase.....	36
Figure 2.6.	Schematic representation of <i>A. americanum</i> cAPK-cDNAs and proteins .....	38

Figure 2.7.	Schematic representation of the prostanoid synthesis pathway.....	39
Figure 2.8.	A schematic representation of the activation of PLC and the role of PIP <sub>2</sub> in intracellular signaling .....	43
Figure 2.9.	Known and hypothesised factors and events controlling secretion in ixodid female salivary glands .....	45
Figure 2.10.	The effect of dopamine and extracellular calcium on apyrase secretion from the salivary glands of <i>O. savignyi</i> .....	50
Figure 2.11.	The effect of isoproterenol on apyrase secretion from the salivary glands of <i>O. savignyi</i> .....	51
Figure 2.12.	The effect of carbachol on apyrase secretion from the salivary glands of <i>O. savignyi</i> .....	52
Figure 2.13.	The effect of intracellular calcium on dopamine-stimulated apyrase secretion from permeabilized salivary glands of <i>O.</i> <i>savignyi</i> .....	52
Figure 2.14.	The effect of OPC on dopamine-stimulated apyrase secretion from permeabilized salivary glands of <i>O. savignyi</i> .....	53
Figure 2.15.	PGE <sub>2</sub> stimulated apyrase secretion from intact salivary glands in the presence of HBSS with calcium .....	54
Figure 2.16.	PGE <sub>2</sub> stimulated apyrase secretion from permeabilized salivary glands in the presence of HBSS with calcium.....	55
Figure 2.17.	Rescue of OPC treated cells with PGE <sub>2</sub> .....	55
Figure 2.18.	The effect of elevated extracellular cAMP levels on apyrase secretion from intact salivary glands of <i>O. savignyi</i> .....	56
Figure 2.19.	The effect of elevated intracellular cAMP levels on apyrase secretion from permeabilized salivary glands of <i>O. savignyi</i> .....	56
Figure 2.20.	The effect of elevated intracellular cAMP levels on dopamine- stimulated apyrase secretion from permeabilized salivary glands of <i>O. savignyi</i> .....	57
Figure 2.21.	The effect of verapamil on dopamine-stimulated apyrase secretion from intact salivary glands .....	58
Figure 2.22.	The effect of Ouabain on dopamine-stimulated apyrase secretion in HBSS with calcium .....	59
Figure 2.23.	The effect of dopamine and extracellular calcium on apyrase secretion from the salivary glands of <i>O. savignyi</i> in HBSS.....	61

Figure 2.24.	The effect of dopamine and extracellular calcium on apyrase secretion from the salivary glands of <i>O. savignyi</i> in AISS.....	61
Figure 2.25.	Effect of N-ethylmaleimide on dopamine-stimulated apyrase secretion .....	62
Figure 2.26.	Effect of GTP $\gamma$ S on apyrase secretion .....	63
Figure 2.27.	Western blotting of dopamine and cAMP treated salivary glands using a monoclonal anti-phosphothreonine IgG .....	64
Figure 2.28.	A schematic presentation of the functions of the various reactions catalyzed by cellular phosphoinositide kinase isozymes .....	66
Figure 2.29.	Effect of Wortmannin on dopamine-stimulated apyrase secretion .....	67
Figure 2.30.	Effect of IP <sub>3</sub> on apyrase secretion from permeabilized salivary glands of <i>O. savignyi</i> .....	68
Figure 2.31.	Effect of U73,122 on dopamine-stimulated apyrase secretion from permeabilized salivary glands of <i>O. savignyi</i> .....	69
Figure 2.32.	The effect of cytochalasin D on dopamine-stimulated apyrase secretion .....	71
Figure 2.34.	The effect of colchicine on dopamine-stimulated apyrase secretion .....	72
Figure 2.35.	Schematic representation of the proposed mechanisms underlying regulated exocytosis of apyrase from LDCVs from the salivary glands of <i>O. savignyi</i> .....	75

### **Chapter 3:**

Figure 3.1.	Model of the ionic layer of the yeast post-Golgi SNARE complex .....	83
Figure 3.2.	Crystal structure of the neuronal Sec1/syntaxin 1a complex .....	84
Figure 3.3.	Protein structure of neuronal SNAP-25 and ubiquitously expressed homologues.....	87
Figure 3.4.	A model for Rab recruitment .....	91
Figure 3.5.	Diagram of the domain structure of synaptotagmin I .....	94
Figure 3.6.	Flow chart of Super SMART™ cDNA synthesis .....	103
Figure 3.7.	Cloning strategy during 3'-RACE .....	105
Figure 3.8.	Identification of SNAREs and Rab3a using Western Blotting .....	110
Figure 3.9.	Identification of a high molecular mass core complex in the salivary glands of <i>O. savignyi</i> .....	111

Figure 3.10.	Immuno-localization of syntaxin in the salivary glands of <i>O. savignyi</i> using anti-rat brain syntaxin2 polyclonal antibodies.....	112
Figure 3.11.	Immuno-localization of VAMP in the acini of <i>O. savignyi</i> using anti-rat brain VAMP2 polyclonal antibodies .....	112
Figure 3.12.	Immuno-localization of VAMP in granular cells of <i>O. savignyi</i> salivary glands.....	113
Figure 3.13.	Immuno-localization of SNAP25 in acini of <i>O. savignyi</i> .....	113
Figure 3.14.	Immuno-localization of actin in acini of <i>O. savignyi</i> .....	114
Figure 3.15.	Immuno-localization of tubulin in the acini of <i>O. savignyi</i> .....	115
Figure 3.16.	Electrophoretic analysis of total RNA.....	115
Figure 3.17.	Agarose electrophoresis of the open reading frame amplified from recombinant synaptotagmin I .....	116
Figure 3.18.	Amino acid similarity among five synaptotagmin isoforms .....	117
Figure 3.19.	3'-RACE with synaptotagmin primer 1 (SDPYVK) and cDNA created from salivary glands of <i>O. savignyi</i> , whole <i>O. savignyi</i> ticks and rat brain (positive control).....	118
Figure 3.20.	3'-RACE with salivary gland RNA and the syt_2 primer .....	119
Figure 3.21.	Hybridisation of the putative synaptotagmin clones obtained with the DIG-labelled sytI probe .....	119
Figure 3.22.	Amino acid sequence alignment of various syntaxins .....	120
Figure 3.23.	PCR amplification of syntaxin using the syn_1 degenerative primer from <i>O. savignyi</i> salivary gland cDNA .....	121
Figure 3.24.	PCR amplification of syntaxin using the syn_1 degenerative primer from <i>Argas (P.) walkerae</i> cDNA.....	121
Figure 3.25.	DNA nucleotide and amino acid sequence of <i>A. walkerae</i> clone obtained with syn_1.....	122
Figure 3.26.	Taguchi-PCR with syn_2 using salivary gland cDNA from <i>O. savignyi</i> .....	123
Figure 3.27.	High Pure isolation of the 500 bp band obtained with syn_2.....	123
Figure 3.28.	Nucleotide and amino acid sequence of the 500 bp band obtained with syn_2 primer from salivary gland cDNA.....	123
Figure 3.29.	Schematic presentation of the suppression PCR effect.....	125
Figure 3.30.	Analysis of ds cDNA amplification by LD-PCR using Super SMART™ technology .....	126

Figure 3.31.	Taguchi_PCR with the syn_1 primer using ds SMART DNA from salivary glands of <i>O. savignyi</i> .....	127
Figure 3.32.	Agarose electrophoresis of the purified 450 bp product obtained with syn_1 and SMART DNA.....	127
Figure 3.33.	Nucleotide and amino acid sequence of 450bp band obtained with syn_1 primer from SMART salivary gland cDNA .....	127
Figure 3.34.	Amino acid sequence alignment of various syntaxin isoforms 2 and 3 .....	128
Figure 3.35.	3'-RACE with the syn_2/3 primer using ds SMART DNA from salivary glands of <i>O. savignyi</i> .....	129
Figure 3.36.	Localization of SNAREs and cytoskeletal proteins in the acini of <i>O. savignyi</i> .....	131

**Chapter 4:**

Figure 4.1.	Schematic diagram of the GAL4-based two-hybrid system.....	138
Figure 4.2.	pAS2-1 map and MCS .....	139
Figure 4.3.	pACT2 map and MCS .....	142
Figure 4.4.	Schematic presentation of a yeast promoter.....	144
Figure 4.5.	Reporter gene constructs in the yeast strains AH109 .....	145
Figure 4.6.	Schematic representation of directional cloning using <i>SfI</i> digestion .....	151
Figure 4.7.	Schematic representation of fragmenting the full-length <i>SfI</i> library using random primers .....	155
Figure 4.8.	Schematic presentation of (A) native syntaxin 1 and (B) truncated syntaxin 1 bait.....	157
Figure 4.9.	Schematic presentation of (A) native Rab3a and (B) mutated Rab3a bait constructs .....	158
Figure 4.10.	Analysis of ds cDNA amplification by LD-PCR using Super SMART™ technology .....	165
Figure 4.11.	Agarose gel electrophoresis of (1) polished ds cDNA and (2) purified <i>SfI</i> digested ds SMART DNA .....	166
Figure 4.12.	Agarose gel electrophoresis of (1) <i>SfI</i> digested pACT2, (2) <i>SfI</i> digested pACT2 treated with T4 Ligase and (3) untreated intact pACT2.....	166

Figure 4.13.	Transformation of various insert: vector ratios into electro competent BL21 <i>E. coli</i> cells .....	167
Figure 4.14.	Agarose gel electrophoresis of <i>Sfi</i> I digested plasmids isolated from GAL4 AD/library transformed BL21 <i>E. coli</i> cells .....	167
Figure 4.15.	Agarose gel electrophoresis of the <i>Xba</i> I digested fragmented dsDNA .....	168
Figure 4.16.	PCR screening of cloned inserts from transformed BL21 <i>E. coli</i> cells .....	169
Figure 4.17.	DNA sequence of four similar molecular mass clones from the fragmented <i>Sfi</i> /XbaI GAL4AD fusion library .....	169
Figure 4.18.	PCR amplification of syntaxin bait constructs .....	170
Figure 4.19.	Amino acid sequence alignment of the syntaxin baits .....	171
Figure 4.20.	ELISA of syntaxin transformed AH109 cells with polyclonal anti-syntaxin 2 IgG .....	171
Figure 4.21.	PCR amplification of the coding region of native mouse brain Rab3a .....	172
Figure 4.22.	ELISA of Rab3a T36N transformed AH109 cells with polyclonal anti-Rab3a IgG .....	172
Figure 4.23.	DNA nucleotide sequence alignment of the various Rab3a bait constructs .....	173
Figure 4.24.	PCR amplification of the coding region of native mouse brain $\alpha$ -SNAP .....	174
Figure 4.25.	DNA nucleotide sequence alignments of $\alpha$ -SNAP bait constructs .....	175
Figure 4.26.	AH109 yeast cells containing the pAs2_1 truncated syntaxin bait construct .....	176
Figure 4.27.	AH109 yeast cells co-transformed with truncated syntaxin bait and <i>Sfi</i> /XbaI truncated library .....	177
Figure 4.28.	AH109 yeast cells containing the pAs2_1 native Rab3a bait construct .....	177
Figure 4.29.	A typical $\beta$ -galactosidase colony lift assay of AH109 yeast cells containing the pAS2_1 truncated syntaxin bait construct .....	178
Figure 4.30.	Partial sequence of the pACT2 plasmid .....	179
Figure 4.31.	Typical agarose electrophoresis pattern obtained after nested PCR of QDO-positive clones containing truncated syntaxin as bait .....	179

Figure 4.32.	Agarose electrophoresis pattern obtained after <i>Bam</i> HI and <i>Hind</i> III digestion of nested PCR products obtained from QDO-positive clones containing truncated syntaxin as bait.....	180
Figure 4.33.	Agarose electrophoresis pattern obtained after <i>Bam</i> HI and <i>Hind</i> III digestion of nested PCR products obtained from QDO-positive clones containing $\alpha$ -SNAP as bait.....	180
Figure 4.34.	Homology between domain I and syntaphilin using PSI-BLAST .....	182
Figure 4.35.	Homology between clone 10 and Casein kinase I epsilon isoform using PSI-BLAST.....	182
Figure 4.36.	Structure prediction of syntaxin interacting peptides .....	184
Figure 4.37.	Crystal structure of syntaxin 1N .....	186
Figure 4.38.	Secondary structure prediction of the $\alpha$ -SNAP interacting protein .....	186
Figure 4.39.	Multiple sequence alignment of syntaxins and $\alpha$ -SNAP interacting protein.....	188
Figure 4.40.	Modeled structure of the $\alpha$ -SNAP interacting protein .....	189
Figure 4.41.	Schematic presentation of a possible model for fusion complex formation in the salivary glands of <i>O. savignyi</i> .....	190

### **Chapter 5:**

Figure 5.1.	Interactions of v- and t-SNAREs in yeast .....	196
Figure 5.2.	Plasmid map of the <i>S. cerevisiae</i> / <i>E. coli</i> shuttle vector pRS 413 .....	199
Figure 5.3.	Putative $\alpha$ -SNAP binding sites on the SNARE complex.....	202
Figure 5.4.	Proposed SNAP-SNARE binding model.....	203
Figure 5.5.	Agarose gel electrophoresis of (i) the ds SMART cDNA synthesized using the <i>Bam</i> H I SMART- and <i>Eco</i> R I CDS primers and (ii) the SMART ds DNA after <i>Bam</i> H I and <i>Eco</i> R I digestion .....	210
Figure 5.6.	Agarose gel electrophoresis of the ds SMART cDNA synthesized using the <i>Sac</i> I SMART- and CDS III primer .....	211
Figure 5.7.	Agarose electrophoresis of the nested PCR products from suppressed H603 cells.....	212
Figure 5.8.	Agarose electrophoresis of the nested PCR products from KC8 cells .....	212
Figure 5.9.	Multiple sequence alignment of syntaxins and knockout suppressor peptides.....	215

Figure 5.10.	Multiple sequence alignments of clone 20 (H603_20) and human syntaxin 1 (1Dn1_B) .....	216
Figure 5.11.	Multiple sequence alignments of clone 27 (H603_27) and human syntaxin 1 (1Dn1_B) .....	216
Figure 5.12.	Secondary structure prediction of the knockout suppressor peptides.....	217
Figure 5.13.	Structure of the Complexin / SNARE Complex.....	217
Figure 5.14.	Modeled structure of the knockout fragment encoded by clone 27 .....	218
Figure 5.15.	ELISA of pull-down eluates using polyclonal antibodies against the various SNAREs and Rab3a.....	219
Figure 5.16.	SDS-PAGE of pull-down eluates .....	219
Figure 5.17.	Multiple sequence alignment of the putative syntaxins isolated from <i>O. savignyi</i> salivary glands .....	221

**LIST OF TABLES****Chapter 1:**

Table 1.1.	Properties of the granule components secreted by argasid ticks .....	5
Table 1.2.	Effects of altered loop-regions in various proteins.....	9
Table 1.3.	Examples of RSPs associated with lipid microdomains .....	11
Table 1.4.	Properties and binding partners of tethering proteins.....	14

**Chapter 2:**

Table 2.1.	General features of female ixodid tick salivary gland acini .....	28
Table 2.2.	General features of the cell types found in the type II acinus of the ixodid tick, <i>R. appendiculatus</i> .....	29
Table 2.3.	General features of the cell types found in the type III acinus of ixodid ticks .....	30
Table 2.4.	Structural classification of dopamine receptors .....	32
Table 2.5.	Structural classification of Protein kinases A / cAMP-dependent kinases .....	37
Table 2.6.	Structural classification of the phospholipases A <sub>2</sub> .....	39
Table 2.7.	Structural classification of prostanoid receptors .....	40
Table 2.8.	Schematic presentation of the micro-titer plate setup in the secretion assay.....	48
Table 2.9.	Molecular masses of proteins phosphorylated by a dopamine- sensitive cAMP-kinase in the salivary glands of the ixodid tick <i>A.</i> <i>americanum</i> and the argasid tick <i>O. savignyi</i> .....	64
Table 2.10.	Characteristics of Type 1A and 1B phophatidylinositol 3- kinases sensitive to Wortmannin.....	66
Table 2.11.	Comparison between the signaling pathways regulating exocytosis from the salivary glands of <i>A. americanum</i> (Ixodidae) and <i>O. savignyi</i> (Argasidae).....	73

**Chapter 3:**

Table 3.1.	Cells with secretory granules .....	80
Table 3.2.	Key proteins that function in exocytosis in neurons and in secretory granule exocytosis.....	82

Table 3.3.	Cellular and functional information about mammalian syntaxins .....	85
Table 3.4.	Cellular and functional information of synaptobrevins.....	86
Table 3.5.	Localization, function and effectors of selected Rab GTPases.....	93
Table 3.6.	Properties of various synaptotagmin isoforms.....	95
Table 3.7.	Properties of the synaptotagmin degenerative primers.....	117
Table 3.8.	Properties of the syntaxin degenerative primers .....	120
Table 3.9.	Properties of the serine protease degenerative primer.....	124
Table 3.10.	Super SMART™ primers used for cDNA synthesis and LD-PCR .....	125
Table 3.11.	Properties of the syn_2/3 degenerative primer .....	129
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 .....	129

**Chapter 4:**

Table 4.1.	MATCHMAKER yeast strain genotypes and applications .....	145
Table 4.2.	The use of various SNAREs and secretory proteins in two-hybrid assays.....	146
Table 4.3.	Primers used for synthesis and amplification of cDNA during cDNA library construction .....	151
Table 4.4.	Ligation of the GAL4 AD / plasmid library using the pACT2 vector (8100 bp).....	152
Table 4.5.	MATCHMAKER yeast strain phenotypes .....	156
Table 4.6.	Primers used for the amplification of native bait constructs .....	157
Table 4.7.	Reverse primer used for the amplification of the syntaxin 1-265 construct.....	158
Table 4.8.	Primers used for the site-directed mutagenesis of Rab3a .....	159
Table 4.9.	Control vectors of the MATCHMAKER™ GAL4 two-hybrid system 2 .....	161
Table 4.10.	Nested PCR primers .....	163
Table 4.11.	Prey molecules identified using truncated syntaxin and truncated library .....	181
Table 4.12.	Predict protein analysis of $\alpha$ -SNAP interacting protein .....	185

**Chapter 5:**

Table 5.1.	Conserved sequence motifs in Ras proteins from different species .....	196
------------	--	-----

Table 5.2.	Properties of the primers used for SMART cDNA synthesis of the <i>Bam</i> HI / <i>Eco</i> R I library .....	209
Table 5.3.	Properties of the primers used for SMART cDNA synthesis of the <i>Sac</i> I / <i>Xba</i> I library .....	210
Table 5.4.	Properties of the SSO-mutated temperature sensitive yeast strains.....	211
Table 5.5.	Deduced amino acid sequence of inserts that suppressed the SSO1 temperature sensitive phenotype of H603 cells.....	213
Table 5.6.	Calculated similarities and identities between identified protein domains and various full-length syntaxin isoforms .....	214

## APPENDIX

Scheme 1:	Overview of performing a yeast two-hybrid screen .....	232
-----------	--	-----

## ACKNOWLEDGEMENTS

I am extremely grateful towards the following:

- Prof. A.W.H. Neitz, my supervisor at the Department of Biochemistry, University of Pretoria, whom inspired my love for biochemistry during the first lecture he presented on proteins during my 2<sup>nd</sup> year undergraduate studies; for opening numerous research opportunities, his continued support, interest and guidance during the duration of my post-graduate life.
- Prof. A.I.Louw, my co-supervisor at the Department of Biochemistry, University of Pretoria, for valuable advice, teaching me to write proper science, continued interest in this project and creating a passion for molecular biology.
- Prof. J.R. Sauer at the Department of Entomology, Oklahoma State University, USA for opening up his laboratory and home to me during my visit. Your Christian values and life will continue to be an inspiration throughout my life.
- Prof. H. Moolman-Smook at the Department of Medical Biochemistry, University of Stellenbosch, South Africa, for opening her laboratory to me, teaching me the art of the two-hybrid system and yeast, your support and valuable opinions.
- Dr. Fourie Joubert and Mr. Tjaart de Beer for their tireless advice on Bioinformatics, computational analyses of data and protein modeling.
- Dr. Ben Mans for the numerous discussions and philosophical talks on life. Your love for ticks inspired me to become a life-long tick person!
- Mrs. S. van Wyngaardt, for her support, advice, helping hands and guidance during this project.
- My fellow students and friends, for always inspiring me to do better!

- My parents, family and friends. Your love, motivation and prayer make life worth living.
- My husband, Nicholas Olivier, who supported me throughout my postgraduate studies. Your kindness, inspiration, guidance, prayer and love are the center of my being.
- The Andrew F. Mellon Foundation for the Mellon Foundation Postgraduate Mentoring Fellowship. This opportunity opened a tremendous amount of opportunities during this study. The scientific exposure I received shaped me into the scientist I am today.
- The National Research Foundation of South Africa for their financial assistance during this study.
- My heavenly Father, thanks for always being the same, unchangeable Rock of my life. Your presence kept me going throughout the good and bad times of this study. I admire your creation, in awe!