NOTE TO USERS

The original manuscript received by UMI contains pages with slanted print. Pages were microfilmed as received.

This reproduction is the best copy available

UMI
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
ABSTRACT

Correct targeting of proteins to specific cellular compartments is thought to occur via transport vesicles. A current hypothesis proposes that in the nervous system, targeting specificity of synaptic vesicles is achieved by interaction of a vesicle protein (VAMP) and presynaptic plasma membrane proteins (syntaxin and SNAP-25). VAMP and syntaxin isoforms have been identified outside the nervous system, but SNAP-25 expression is restricted to neural and neuroendocrine cells. I hypothesized that functional equivalent(s) of SNAP-25 must exist in non-neural cells to allow intracellular traffic. This Thesis describes the search for a non-neural equivalent of SNAP-25, SNAP-23, by screening a melanoma cDNA library. cDNA clones were isolated, sequenced, and fusion proteins were expressed. With a specific antibody, SNAP-23 was detected in all non-neural tissues and cell lines examined. Subcellular fractionation of 3T3-L1 adipocytes suggested that SNAP-23 was predominantly found at the plasma membrane.
PREFACE

The work presented in this Thesis was performed from 1995-1997 in the laboratory of Dr. Amira Klip, Division of Cell Biology, The Research Institute of the Hospital for Sick Children, Toronto, Ontario, Canada with the financial support of the University of Toronto (University of Toronto Open Fellowship) and a grant from the Medical Research Council to Dr. Amira Klip.

The results of this Thesis have been presented in two publications:


TABLE OF CONTENTS

ABSTRACT........................................................................................................ ii
PREFACE........................................................................................................ iii
TABLE OF CONTENTS...................................................................................... iv
LIST OF TABLES.............................................................................................. vi
LIST OF FIGURES............................................................................................ vii
LIST OF ABBREVIATIONS................................................................................ viii

BACKGROUND.................................................................................................. 1
The SNARE Hypothesis: a Conceptual Framework for Studying Intracellular Traffic......................................................... 1

Part I: Components of the SNARE hypothesis........................................... 2
Soluble Proteins................................................................................................. 2
  NSF.................................................................................................................. 2
SNAPs................................................................................................................. 4
Membrane-associated Proteins................................................................. 6
t-SNAREs.......................................................................................................... 7
  Syntaxins......................................................................................................... 7
SNAP-25........................................................................................................... 11
v-SNAREs........................................................................................................ 16
VAMPs............................................................................................................. 16
Synaptotagmins............................................................................................... 19

Part II: Models for Vesicle Targeting, Docking and Fusion................. 28
Vesicle Targeting and Docking...................................................................... 28
  The SNARE hypothesis: a general vesicle targeting and docking model.......................... 28
Recent modifications to the SNARE hypothesis.................................. 32
Membrane Fusion............................................................................................ 36

Part III: Proteins Modulating the Accessibility of the SNAREs........ 42
(1) n-Sec1 / Munc18 / rbSec1........................................................................ 42
(2) Rab............................................................................................................ 44

Part IV: Role of SNAREs in Insulin-stimulated Glucose Transport........ 48
v-SNAREs .......................... 53
VAMPs .................................. 53
Synaptotagmins ......................... 54
t-SNAREs ................................ 55
Syntaxins ................................ 55
SNAP-25 .................................. 56

RATIONALE AND HYPOTHESIS .................. 57
IDENTIFICATION OF A SNAP-25 LIKE PROTEIN IN NON-NEURAL CELL LINES AND TISSUES .................. 58

Introduction .................................. 58
Experimental Procedures .................. 59
Materials ..................................... 59
Methods ..................................... 59
RNA isolation and Northern blot analysis .......... 59
cDNA cloning and sequencing ................. 60
Bacterial expression of SNAP-23 and SNAP-25 proteins .... 61
Generation of SNAP-23 antisera ............... 61
Cell culture ................................... 61
Membrane isolation ............................ 62
Subcellular fractionation of 3T3-L1 adipocytes ...... 63
Immunoblotting ................................. 63

Results ........................................ 65
Isolation of SNAP-23 from human melanoma cDNA library ...... 65
Generation of two SNAP-23 specific antibodies .......... 67
Expression of SNAP-23 protein in different cell lines and tissues .... 67
Subcellular localization of SNAP-23 in 3T3-L1 cells .......... 68
Subcellular distribution of SNAP-23 in non-neural secretory cells .... 69

Discussion ..................................... 82
SNAP-23 is a SNAP-25-related gene product ............ 82
Expression of SNAP-23 in different tissues and cells .......... 85
Subcellular localization of SNAP-23 in non-neural cells .......... 86

OVERALL DISCUSSION AND FUTURE DIRECTIONS .......... 91
REFERENCES ................................... 97
APPENDED PAPERS ............................. 117
# LIST OF TABLES

**Table B1**  Similarity between the subcellular localization of proteins involved in the nerve-terminal and yeast transport machinery ........................................ 25

**Table B2**  Similarity between the nerve-terminal and yeast membrane traffic machinery ........................................................................................................... 26

**Table B3**  A summary of molecules that interact with different tagmin isoforms...... 27
LIST OF FIGURES

BACKGROUND

Fig. B1 Schematic representation of the predicted coiled-coil domains of SNAREs and α-SNAP which mediate protein-protein interactions.............................................. 23

Fig. B2 Schematic representation of the positions of cleavage of SNAREs by different Clostridial neurotoxins................................................................. 24

Fig. B3 Proposed model for the docking and fusion of synaptic vesicles to the presynaptic plasma membrane at a nerve terminal.................................................. 31

Fig. B4 Two proposed models for synaptic vesicle docking and fusion.............. 35

Fig. B5 Scaffold model for membrane fusion during exocytosis.................... 41

IDENTIFICATION OF A SNAP-25 LIKE PROTEIN IN NON-NEURAL CELL LINES AND TISSUES

Fig. 1 A SNAP-25 cDNA probe did not hybridize with RNA from 3T3-L1 cells...... 72

Fig. 2 Immunodetection of a SNAP-25 like protein in membranes from 3T3-L1 adipocytes........................................................................................................... 73

Fig. 3 Nucleotide sequence of human melanoma SNAP-23 and its predicted amino acid sequence................................................................. 74

Fig. 4 Comparison of amino acid sequences of human melanoma SNAP-23 and SNAP-25B........................................................................................................ 75

Fig. 5 Characterization of SNAP-23 and SNAP-25 antibodies.............................. 76

Fig. 6 Expression of SNAP-23 in cell lines and tissues..................................... 77

Fig. 7 Localization of SNAP-23 in 3T3-L1 cells............................................ 78

Fig. 8 Expression of SNAP-23 in different types of white blood cells............. 79

Fig. 9 Subcellular distribution of SNAP-23 in human neutrophils.................... 80

Fig. 10 Distribution of SNAP-23 in pancreatic acinar cells............................. 81
LIST OF ABBREVIATIONS

ADP  Adenosine diphosphate
Ala  Alanine
Arg  Arginine
ATP  Adenosine triphosphate
BoNT  Botulinum neurotoxin
Ca$^{2+}$  Calcium
cDNA  Complementary deoxyribonucleic acid
CNS  Central nervous system
CYT  Cytosol
EDTA  Ethylenediamine tetraacetic acid
ER  Endoplasmic reticulum
EST  Expressed sequence tag
Gln  Glutamine
GLUT  Glucose transporter
GST  Glutathione-S-transferase
Hepes  N-[2-Hydroxyethyl] piperazine-N' [2-ethanesulfonic acid]
IDDM  Insulin-dependent diabetes mellitus
Ile  Isoleucine
IRS-1  Insulin receptor substrate-1
Kb  Kilobase
kDa  Kilodalton
LDM  Light density microsomes
Lys  Lysine
Mg  Magnesium
mM  Millimolar
mRNA  Messenger ribonucleic acid
Mw  Molecular weight
NEM  N-ethylmaleimide
NIDDM  Non-insulin-dependent diabetes mellitus
NSF  NEM-sensitive factor
PC12  Rat pheochromocytoma cell line
PCR  Polymerase chain reactions
PDGF  Platelet-derived growth factor
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Acronym/Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Phosphohistidine</td>
<td></td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inorganic orthophosphate</td>
<td></td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
<td></td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
<td></td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
<td></td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology-2</td>
<td></td>
</tr>
<tr>
<td>SNAP</td>
<td>Soluble NSF attachment protein</td>
<td></td>
</tr>
<tr>
<td>SNAP-23</td>
<td>Synaptosomal-associated protein of 23 kDa</td>
<td></td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Synaptosomal-associated protein of 25 kDa</td>
<td></td>
</tr>
<tr>
<td>SNARE</td>
<td>SNAP Receptor</td>
<td></td>
</tr>
<tr>
<td>TeTx</td>
<td>Tetanus toxin</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
<td></td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle-associated membrane protein</td>
<td></td>
</tr>
<tr>
<td>ZGM</td>
<td>Zymogen granule membrane</td>
<td></td>
</tr>
</tbody>
</table>
THE SNARE HYPOTHESIS: A CONCEPTUAL FRAMEWORK FOR STUDYING INTRACELLULAR TRAFFIC

A fundamental question in cell biology is how are proteins targeted to specific cellular compartments by means of transport vesicles. The transport machinery is crucial for generation of specific organelles such as the plasma membrane, endosomes and Golgi stacks, as well as maintaining their compartmental integrity. This would require the constitutive transport of vesicles to and from their destined compartments. Another aspect of the life cycle of a cell is its ability to respond to extracellular stimuli which involves regulated exocytic processes. Regulated exocytosis is responsible for cellular events such as neurotransmitter release, hormone secretion, and glucose transport.

Recent findings from five lines of study in different organisms using molecular biology techniques, biochemical and pharmacological approaches, suggest a universal docking and fusion machinery that is conserved in both prokaryotic and eukaryotic cells (reviewed in Sollner and Rothman, 1994). These studies are as follows: (1) cell free transport assays between Golgi stacks led to the identification of two essential general components of the transport mechanism which are referred to as NSF and SNAPs; (2) the subsequent identification of proteins that serve as receptors for SNAPs (SNAREs) on both the vesicles (v-SNAREs) and the target membranes (t-SNAREs) in the nervous system; (3) the discovery that some SNAREs are specific proteolysis substrates of different clostridial neurotoxin serotypes and the observations that cleavage results in abrogation of neurotransmitter release highlighted the importance of SNAREs in regulated secretion; (4) the discovery of homologues or isoforms of different docking and/or fusion proteins in non-neural cells of eukaryotes; (5) the existence of related gene products in yeast and the isolation of lethal yeast mutants defective in various constitutive transport steps. Results from these five different lines of study suggest that the molecular mechanism for vesicle
targeting, docking and fusion is conserved from yeast to mammals; moreover, the machinery is similar between constitutive and regulated intracellular transport.

As mentioned above, the vesicle docking and/or fusion apparatus involves general components such as NSF and SNAPs which are both soluble proteins, as well as membrane proteins that are localized on either the target membranes (t-SNAREs) or the vesicles (v-SNARE). I have organized this section into four parts: part I will present findings on each of the components of the docking/fusion complex; part II will introduce a model known as the "SNARE hypothesis" which was originally proposed by Rothman and colleagues to explain how synaptic vesicles dock and fuse with the presynaptic plasma membrane leading to neurotransmitter release (Rothman, 1994). Part III will discuss two proteins that may have a regulatory role in the docking/fusion machinery by modulating the accessibility of the SNAREs. Part IV will introduce the function of SNAREs in the regulated exocytic process facilitating glucose transport in muscle and fat cells.

**Part I: Components of the SNARE Hypothesis**

**Soluble Proteins**

**NSF**

The establishment of a cell-free assay by reconstituting vesicular transport between cisternae of Golgi membranes has been useful in elucidating the molecular mechanisms underlying constitutive protein transport pathways. The finding that vesicular transport between Golgi stacks *in vitro* is blocked by the cysteine-alkylating agent N-ethylmaleimide (NEM) resulting in an accumulation of uncoated vesicles at the acceptor Golgi cisternae, suggests that a NEM-Sensitive Factor (NSF) is needed for fusion (Malhotra et al., 1988; Orci et al., 1989). Here, fusion is defined as any steps leading to mixing of the donor (vesicles) and acceptor (Golgi stacks) compartments once the transport vesicle binds to its target (Rothman, 1994). Moreover, the blockade can be rescued upon addition of fresh
observations together led to the purification of NSF from the cytosol (Block et al., 1988).

NSF is a homotrimer of 76 kDa subunits. Each of the subunits contains 2 homologous ATP-binding domains (Wilson et al., 1989; Tagaya et al., 1993). ATP hydrolysis by the first ATP-binding domain is required for NSF function in intra-Golgi transport. Mutations of residues within nucleotide-binding motifs result in only 20-30% of wild type ATPase activity and no intra-Golgi transport activity, although mutated proteins can still form homotrimer (reviewed in Whiteheart and Kubalek, 1995). Furthermore, only normal trimers are active in Golgi transport; trimers with even one mutated subunit are inactive, indicating that all three subunits work in concert. The second ATP-binding domain is needed for trimerization since its deletion results in monomeric NSF. Mutations of the nucleotide-binding motifs of this second domain result in mutant proteins supporting up to 30% of intra-Golgi transport in vitro (Whiteheart and Kubalek, 1995). Therefore, the role of this second ATP-binding site, other than for trimerization of subunits, remains unclear.

Two pieces of direct evidence that NSF is required in vesicle transport in vivo were obtained by isolating mutant yeast and flies that bear mutations in their NSF homologues. First, in the absence of yeast NSF homologue, Sec18p, uncoated vesicles accumulate at the acceptor membrane (Malhotra et al., 1988; Orci et al., 1989). Moreover, loss of Sec18p function has been demonstrated to be lethal (Eakle et al., 1988; Kaiser and Schekman, 1990). These results indicate that Sec18p is required for transport vesicle fusion in yeast. Second, in Drosophila, the comatose mutant has a temperature-sensitive mutation in NSF which leads to a block in neurotransmission, provides a direct functional evidence for a role of NSF in regulated exocytosis (Pallanck et al., 1995). Switching these flies to the restrictive temperature results in a rapid (within 1-2 min) inhibition of neurotransmission and paralysis, consistent with an essential role for NSF in synaptic exocytosis.
Studies using different cell-free systems show that NSF is required for other intracellular transport events such as endosomal fusion, endoplasmic reticulum to Golgi transport, and regulated exocytosis of synaptic vesicles. The yeast homologue of NSF, Sec18p, is also involved in many different fusion events with two exceptions: fusion of haploid nuclei (karyogamy) and transport between the Golgi complex and the vacuole (reviewed in Whiteheart and Kubalek, 1995). Together these findings suggest that NSF is a general component in the vesicle transport machinery. In addition, it has been observed that plant cytosol can fully replace animal cytosol in cell-free Golgi transport, and that yeast Sec18p can replace NSF in a mammalian system for cell-free Golgi transport (Wilson et al., 1989) which strongly supports the universality of the fusion machinery in vesicle docking.

Although there is an ample body of evidence suggesting the importance of NSF in vesicle transport, the precise step at which NSF acts remains highly controversial. Evidence exists in supporting its action in at least three different stages of vesicle traffic, including a post-docking stage close to membrane fusion (Sollner et al., 1993a), a post-docking but pre-fusion stage (O’Connor et al., 1994), or at a pre-docking stage (Morgan and Burgoyne, 1995a). Future experiments are needed to address the discrepancy among these studies.

**SNAPs**

NSF itself does not bind to Golgi membranes unless a crude cytosolic fraction is added (Weidman et al., 1989). This observation suggests the presence of another cytosolic factor that acts as an 'adaptor' for the association of NSF with the Golgi membranes. Three Soluble NSF Attachment Proteins, termed α-, β-, and γ-SNAP (of 35, 36, and 39 kDa respectively), were purified from bovine brain extracts (Clary and Rothman, 1990b). NSF binds only to SNAPs that are already bound to membranes, and not to those that are
induces a conformational change in the SNAPs which exposes the NSF binding site (Wilson et al., 1992).

The three SNAPs have distinct tissue distribution. β-SNAP is expressed mainly in brain whereas α- and γ-SNAP have been found in many cell types. Since α- and β-SNAP have 83% sequence identity (Whiteheart et al., 1993), they are proposed to represent functionally similar isoforms. Indeed, they were found to have interchangeable roles in many aspects of regulated exocytosis, including their ability to bind and activate the ATPase activity of NSF (Rothman, 1994; Sudlow et al., 1996). Furthermore, exogenous α- and β-SNAP stimulated catecholamine release in response to Ca\(^{2+}\) from digitonin-permeabilized chromaffin cells to the same extent and with essentially identical dose-response relationships. Comparative analysis of the role of α-, β- and γ-SNAP in intra-Golgi transport suggested that α- and β-SNAP are functionally redundant isoforms but γ-SNAP has distinct roles (Whiteheart et al., 1992; Wilson et al., 1992). This is consistent with the high level of sequence identity between α- and β-SNAP compared to the divergent γ-SNAP, as it shows only 25% identity to α- and β-SNAP (Whiteheart et al., 1993). γ-SNAP is not essential for NSF binding to Golgi membranes, but synergizes with α-SNAP to create an optimum binding site for NSF. Moreover, γ-SNAP has been shown to have minor and variable effect on catecholamine release (Morgan and Burgoyne, 1995b).

cDNAs for five SNAPs have been cloned: three bovine forms (Clary and Rothman, 1990b), one from Drosophila (Ordway et al., 1994), and one from yeast (Griff et al., 1992). Sec17p (of 34 kDa) is the yeast homologue (with 34% identity) to α-SNAP, since it is α-SNAP (but not β- or γ-SNAP) that can restore the fusion activity of cytosolic extracts from yeast Sec17 mutants (Clary et al., 1990a). This suggests that yeast Sec17p is the functional equivalence to mammalian α-SNAP. Moreover, this finding supports once again the theme of universality in the fusion machinery.
The functional importance of α- and β-SNAP in regulated exocytosis has been supported by three important findings. First, exogenous α-SNAP stimulates exocytosis following its injection into the squid giant synapse (DeBello et al., 1995). Second, addition of recombinant α-SNAP (Chamberlain et al., 1995; Morgan and Burgoyne, 1995b; Sudlow et al., 1996) or β-SNAP (Sudlow et al., 1996) resulted in a stimulation of Ca²⁺-dependent catecholamine release from digitonin-permeabilized adrenal chromaffin cells. Third, the importance of mammalian α-SNAP is underscored by the requirement of its functional equivalent Sec17p for the transit through the secretory pathway in yeast (Pryer et al., 1992). In the absence of a functional Sec17p, transport from endoplasmic reticulum to Golgi stops and transport vesicles accumulate (Kaiser and Schekman, 1990).

**Membrane-associated Proteins**

The SNARE hypothesis postulates that targeting of intracellular vesicles to specific compartment is achieved through the unique interaction of membrane proteins both on the vesicles and the target membranes. These membrane proteins, termed as SNAREs, act as 'receptors' for the general fusion particles, SNAPs and NSF, which have been shown to be necessary for membrane fusion.

When brain extracts were solubilized with detergent, a multisubunit particle of NSF-SNAP-SNARE which sedimented at 20S was isolated (Wilson et al., 1992). In order to purify SNAREs in quantity from crude detergent extract of bovine brain, the formation of the 20S particle was further promoted to assemble in vitro using recombinant NSF, α- and γ-SNAP (Sollner et al., 1993b). Three membrane proteins were identified which were later found to form a stable stoichiometric complex that sedimented at 7S in the absence of the fusion factors NSF and SNAPs (Sollner et al., 1993a). The complex included two presynaptic plasma membrane proteins: SNAP-25 (SyNaptosomal-Associated Protein of 25 kDa) and syntaxin 1. These two proteins are often referred to as
SNAP-25 and soluble protein SNAP represent unrelated gene products which unfortunately share similar names. The complex also contained a synaptic vesicle protein VAMP (vesicle-associated membrane protein, also known as synaptobrevin), which is often referred to as a v-SNARE. Recently, another synaptic vesicle protein, synaptotagmin, has also been described as a v-SNARE based on its ability to bind a neural-specific form of SNAP protein, β-SNAP (Schiavo et al., 1995a) and the two t-SNAREs, syntaxin 1 and SNAP-25 (Schiavo et al., 1997). The four SNARE proteins are described in detail below.

**t-SNAREs**

**Syntaxins**

Syntaxins were first identified as two 35 kDa proteins (p35) that interact with the synaptic vesicle protein p65 or synaptotagmin (Bennett et al., 1992). The two proteins were termed syntaxins 1A and 1B, and found to share 84% sequence identity. So far, seven mammalian syntaxins have been described: 1A, 1B, and 2-6. Although the syntaxins have distinct tissue distribution and subcellular localization, they nonetheless exhibit a few common structural features. All syntaxins are 288-301 amino acids in length (Bennett et al., 1993; Dascher et al., 1994) except syntaxin 6 which is composed of 255 residues (Bock et al., 1996). Furthermore, all members of the syntaxin family have a C-terminal stretch of 17-25 highly hydrophobic residues which is of sufficient length and hydrophobicity to serve as membrane anchor (Bennett et al., 1993). As a consequence of the C-terminal stretch of hydrophobic residues, all syntaxins behave as type II integral membrane proteins with their N-termini facing the cytoplasm (Bennett et al., 1992; Bennett et al., 1993; Bock et al., 1996).
Each member of the syntaxin family contains several domains predicted to form α-helical coiled-coil structures (Inoue et al., 1992; Spring et al., 1993; Bock et al., 1996) which are likely to be involved in protein-protein interactions (Fig. B1). One of such coiled-coils which spans 70 residues near the C-terminal transmembrane region (Kee et al., 1995), also referred to as the H3 domain, displays high level of sequence similarity within the rat syntaxin family (Bennett et al., 1993) and between yeast and rat proteins (Pelham, 1993). *In vitro* binding studies of recombinant proteins revealed that this coiled-coil region of syntaxin 1A (residues 199-243) and syntaxin 4 (amino acids 197-274), but not of syntaxins 2 and 3, interacts directly with VAMPs 1 and 2 (Calakos et al., 1994). The observation that VAMPs interact with some syntaxin members but not others supports the hypothesis that specific SNARE interactions may contribute to vesicle targeting specificity. The H3 domain of syntaxin 1A has also been shown to bind to another t-SNARE, SNAP-25 (Chapman et al., 1994), and soluble protein α-SNAP (Kee et al., 1995) as shown in Fig. B1. Moreover, the H3 domain with the transmembrane region of syntaxin 1A (residues 194-288) binds to synaptotagmin in a Ca\(^{2+}\)-dependent manner (Chapman et al., 1995). Syntaxin 1A has been shown to associate with different types of Ca\(^{2+}\) channels including the N-type voltage-gated Ca\(^{2+}\) channel (Bennett et al., 1992; Sheng et al., 1994; Sheng et al., 1996; Wiser et al., 1996). This interaction predominantly involves the transmembrane region of syntaxin (Bezprozvanny et al., 1995; Wiser et al., 1996). In these studies, co-expression of syntaxin 1A with N- and L-type Ca\(^{2+}\) channels in *Xenopus* oocytes have been shown to exert a negative regulation on both channels. Syntaxin has also been shown to interact with the P- and Q-type Ca\(^{2+}\) channels which triggers rapid release of neurotransmitter at many mammalian synapses (Martin-Moutot et al., 1996).

As mentioned above, a family of seven syntaxin-related proteins in rat have been identified and they share 23-84% amino acid identity. Syntaxins 1A and 1B are expressed in the nervous system (Bennett et al., 1992; Bennett et al., 1993; Parpura et al., 1995), and
endocrine cells including the pancreatic insulin-secreting \( \beta \)-cells (Wheeler et al., 1996), and adrenal chromaffin cells (Bennett et al., 1993; Hodel et al., 1994; Roth and Burgoyne, 1994; Tagaya et al., 1995; Hohne-Zell and Gratzl, 1996). Syntaxins 2-6 are widely expressed in different tissues which supports the hypothesis that this family of proteins participates in vesicle traffic in various cell types. Unique subcellular localization of some syntaxin isoforms may provide the targeting specificity in intracellular traffic. For instance, immunofluorescent detection of transiently expressed isoforms of syntaxin suggests that syntaxins 1A, 2 and 4 are predominantly localized on the plasma membrane in COS cells (Bennett et al., 1993). Furthermore, in polarized exocrine acinar cells of the pancreas, localization of endogenous syntaxins 2 and 4 is restricted to apical and basolateral plasma membrane respectively. Syntaxin 3 was found to be distributed in an intracellular vesicular compartment, the zymogen granule membrane (Gaisano et al., 1996). Low et al. demonstrated that in a different type of polarized epithelial cells, the Madin-Darby canine kidney cells, syntaxins 2, 3, and 4 were expressed. Moreover, when these cells were transfected with different syntaxin isoforms, syntaxins 3 and 4 were restricted to the apical and basolateral plasma membrane respectively, whereas syntaxin 2 was found on both membranes (Low et al., 1996). The mammalian syntaxin proteins share 51-53% similarity with two yeast proteins, Sso1p and Sso2p, which are required for traffic between the Golgi complex and plasma membrane in yeast (Pryer et al., 1992). In addition, syntaxin 5 which is present on the cis-Golgi has been demonstrated to be essential in ER to Golgi transport (Dascher et al., 1994). This is further supported by the finding that the yeast \( SED5 \) gene product, homologue of syntaxin 5 (35% identity), is required for efficient membrane transport between ER and the Golgi complex in yeast (Hardwick and Pelham, 1992). The most recently identified member of the syntaxin family, syntaxin 6, was identified based on its sequence similarity to yeast Pep12p (56% amino acid similarity) (Bock et al., 1996). In yeast, Pep12p is necessary for the delivery of proteolytic enzymes from the Golgi complex.
to the vacuole (Jones, 1978). In rat hepatoma cells, syntaxin 6 is predominantly localized to the Golgi region. It remains to be defined at which step syntaxin 6 participates in intracellular vesicle traffic. Table B1 summarizes the subcellular localization of different syntaxin isoforms. The transport step at which each isoform functions is listed in Table B2.

The function of syntaxin has been examined using various approaches. Microinjection of anti-syntaxin 1A antibodies into PC12 cells results in an inhibition in Ca\(^{2+}\)-regulated catecholamine release (Bennett et al., 1993) which suggests that syntaxin 1A is an important component of the regulated secretory machinery in PC12 cells. A pharmacological approach to understand the function of syntaxin was also employed when syntaxin was found to be cleaved by Botulinum neurotoxin serotype C (BoNT/C) which belongs to a large family of zinc-dependent endopeptidases (Blasi et al., 1993b; Schiavo et al., 1995b). Fig. B2 illustrates the cleavage of SNARE proteins by different clostridial neurotoxins. BoNT/C cleaves at Lys-Ala bond of syntaxins 1A and 1B, only when they are inserted into a lipid bilayer. Syntaxins 2 and 3 are also cleaved by BoNT/C, while syntaxin 4 is resistant (Blasi et al., 1993b; Schiavo et al., 1995b). Cleavage of syntaxins by BoNT/C results in an inhibition of neurotransmitter release (Blasi et al., 1993b), causes growth cone collapse, inhibits axonal growth (Igarashi et al., 1996), and blocks Ca\(^{2+}\)-evoked catecholamine release from intact and permeabilized adrenal chromaffin cells. Moreover, BoNT/C cleavage of syntaxin has recently been shown to uncouple the down-regulation of presynaptic Ca\(^{2+}\) channel by G proteins (Stanley and Mirotznik, 1997). This finding may suggest an additional new role for syntaxin in G-protein modulation of presynaptic calcium channel. Finally, a Drosophila mutant without syntaxin 1A shows subtle axonal defects and partial defects of CNS condensation (Schulze et al., 1995). These results suggest that in addition to a role in secretion, syntaxins participate in phenomena ranging from axon growth to modulation of excitable channel activity.
SNAP-25

SyNaptosomal-Associated Protein of 25 kDa (SNAP-25) is comprised of 206 amino acids with a calculated molecular mass of 23.315 kDa and a theoretical pI of 4.38 (Oyler et al., 1989). In contrast to the well-defined transmembrane domains of other SNAREs, such as syntaxin, amino acid sequence analysis of SNAP-25 suggests that the polypeptide is largely hydrophilic and lacks any stretch of hydrophobic residues necessary for membrane insertion. Rather unexpectedly, characterization of the subcellular localization of SNAP-25 in neurons revealed that the polypeptide is predominantly associated with the synaptosomal membrane fraction, with undetectable levels in the cytosol (Oyler et al., 1989). Furthermore, fractionation studies demonstrated that the association of SNAP-25 with membranes was resistant to 1M salt extraction. The protein was released into the supernatant only after solubilizing the membranes with 1.25% Triton X-100 in the presence of 1M NaCl (Oyler et al., 1989). The tight association of SNAP-25 with the presynaptic plasma membrane was later found to be achieved through a post-translational addition of palmitic acids to one or more of a cluster of 4 cysteine residues spanning amino acids 84-92, which has been referred to as "a cysteine quartet", via thioester linkages (Oyler et al., 1989; Hess et al., 1992). Mutant SNAP-25 with a deletion of 12 amino acids spanning the "cysteine quartet" can no longer be labelled with radioactive palmitate (Veit et al., 1996). Moreover, the deletion renders the protein completely soluble. These two findings further support the hypothesis that SNAP-25 is palmitoylated and that this post-translational modification of the protein is necessary for membrane association.

SNAP-25 was also shown to be substrate for phosphorylation by protein kinase C (PKC) both in vitro and in chromaffin PC12 cells (Shimazaki et al., 1996). The site of phosphorylation is believed to be Ser$^{187}$, although this remains to be confirmed. In addition, it was found that the amount of syntaxin co-precipitated with SNAP-25 decreased
with PKC phosphorylation, suggesting that phosphorylation of SNAP-25 may play a regulatory role, more specifically by blocking the syntaxin-SNAP-25 interaction.

Cloning of the chicken gene for SNAP-25 revealed a complex organization of nine different exons which span more than 65 kilobases of genomic DNA (Bark, 1993). Furthermore, two similar but distinct variants of exon 5 were discovered in chicken (Bark, 1993), mouse (I. Bark and M. Wilson, unpublished), and human (Bark and Wilson, 1994). The alternative splicing of the gene gives rise to two variants: SNAP-25a and SNAP-25b (Bark, 1993). At the amino acid level, the two alternative exon 5 sequences differ in 9 out of 39 possible residues, resulting in two similar but distinct forms of SNAP-25 that share 95% sequence identity (Bark, 1993). Although both isoforms retain the four cysteine residues, the spatial organization with respect to the rest of the sequence has changed. This may affect the efficiency of fatty acid acylation of the polypeptide. Studies on the expression of SNAP-25 isoforms revealed developmental regulation: SNAP-25a is the predominant species during embryonic and early postnatal development of mouse brain, whereas SNAP-25b expression begins with the onset of synaptogenesis and it becomes by far the predominant isoform in the adult brain (Bark et al., 1995). The distribution of SNAP-25 is restricted to neural and neuroendocrine cells including neurons (Oyler et al., 1989), the peripheral nervous system (Duc and Catsicas, 1995), anterior pituitary cells (Aguado et al., 1996), and pancreatic β cells (Jacobsson et al., 1994; Sadoul et al., 1995; Wheeler et al., 1996). SNAP-25 has not been detected in other non-neural secretory cells such as neutrophils (Brumell et al., 1995) and pancreatic exocrine cells (Gaisano et al., 1997).

Like syntaxin, SNAP-25 contains several domains that have α-helical potential, and these predicted secondary structures have been implicated in protein-protein interactions (Fig. B1). For instance, the amino-terminal region has been predicted to contain two coiled-coils (residues 1-42 and 45-85), and both are crucial determinants in its binding to
SNAP-25 also contains a predicted coiled-coil domain at its C-terminus (residues 160-205), which is essential for binding of VAMP (Chapman et al., 1994). In vitro binding studies demonstrated that binding of SNAP-25 greatly increases the affinity between neuronal expressed syntaxin and VAMP proteins, resulting in the formation of a stable SDS-resistant complex (Hayashi et al., 1994; Pevsner et al., 1994a). This suggests that SNAP-25 plays a unique role in strengthening the association of syntaxin and VAMP, to bridge opposing membranes and thereby specify targeting and docking of synaptic vesicles for fusion at the active zone in presynaptic nerve terminals (Wilson et al., 1996). Recently, another v-SNARE, synaptotagmin has also been demonstrated to interact, both in vitro and in vivo, with SNAP-25 (Schiavo et al., 1997). Furthermore, SNAP-25, along with syntaxin and VAMP, has also been shown to associate with N-type calcium channel (Saisu et al., 1991; Sheng et al., 1996) in a Ca\(^{2+}\)-dependent manner. This finding supports the idea that synaptic vesicles are docked near presynaptic Ca\(^{2+}\) channels through this interaction.

Other than mouse, human and chicken, SNAP-25 has also been reported in goldfish, which has two forms of SNAP-25: SNAP-25A and SNAP-25B, due to an ancient gene duplication in bony fish. The two forms are 91% identical to each other, and 94% and 91% identical to mouse SNAP-25b (Risinger and Larhammar, 1993b). SNAP-25 has also been identified in fruitfly Drosophila melanogaster and ray Torpedo marmorata (Risinger et al., 1993a). SNAP-25 sequence is well conserved among species, as its amino acid sequence identity between mouse and ray is 81%, and between mouse and fruitfly is 61%. The product of SEC9 gene is the yeast counterpart of SNAP-25 (Brennwald et al., 1994). SEC9 was originally identified as one of the ten late-acting SEC genes required for post-Golgi transport (Novick et al., 1981) (Table B2). SEC9 gene encodes a hydrophilic 651-residue polypeptide with predicted molecular mass of 73.628 kDa. The predicted Sec9p amino acid sequence revealed no membrane-spanning domain,
consistent with the structure of SNAP-25. The optimal alignment between Sec9p and mouse SNAP-25 protein shows 19% identity and 60% similarity in a 206-residue overlap. Furthermore, expression of the SNAP-25-like domain of Sec9p was found to be sufficient to rescue the temperature-sensitive defect in invertase secretion of yeast mutant sec9-4ts (Brennwald et al., 1994). Subcellular fractionation studies and immunofluorescence staining indicate that the majority of Sec9p is localized to the plasma membrane (Table B1). Unlike SNAP-25, Sec9p entirely lacks cysteine residues and can efficiently be stripped off the membranes by alkaline treatments but not by high salt concentrations. These data suggest that Sec9p is a tightly associated peripheral membrane protein in yeast. Similar to SNAP-25 which has been shown to form a complex with syntaxin and synaptobrevin, Sec9p has also been found to associate with yeast homologues of syntaxin, Sso1p and Sso2p, as well as with the yeast VAMP homologues, Snc1p and Snc2p. This once again supports the hypothesis that the docking and fusion apparatus is conserved from yeast to mammals.

Like syntaxin, SNAP-25 is a target substrate for three Botulinum neurotoxin (BoNT) serotypes: A, E, and C (Schiavo et al., 1993; Blasi et al., 1993a; Binz et al., 1994; Lawrence et al., 1994; Boyd et al., 1995; Pellegrini et al., 1995; Schmidt and Bostian, 1995; Foran et al., 1996) (Fig. B2). There has been one study describing the susceptibility of SNAP-25 to cleavage by BoNT/C which was initially identified as the endopeptidase that targets syntaxin. However, the cleavage site of BoNT/C on SNAP-25 remains to be defined. In contrast, the cleavage sites for BoNT/A and BoNT/E were well characterized. BoNT/A catalyses the hydrolysis of the Gln\textsuperscript{197}-Arg\textsuperscript{198} bond, while BoNT/E cleaves the Arg\textsuperscript{180}-Ile\textsuperscript{181} peptide linkage, generating soluble peptide fragments of 9 and 26 amino acids in length respectively (Schiavo et al., 1993; Binz et al., 1994; Lawrence et al., 1994). Cleavage of SNAP-25 by either type A or E results in an inhibition of neurotransmitter release from isolated nerve terminals (Blasi et al., 1993a), a blockade in
the potassium-stimulated, calcium-dependent insulin release from pancreatic β cells (Boyd et al., 1995), as well as an inhibition on catecholamine secretion by adrenal chromaffin cells (Gutierrez et al., 1995). SNAP-25 antibodies and peptides that contain the cleaved fragment by BoNT/A at the carboxyl-terminal domain of SNAP-25 inhibit Ca²⁺-evoked glutamate release by synaptosomes (Mehta et al., 1996), and block Ca²⁺-dependent catecholamine release from digitonin-permeabilized chromaffin cells (Gutierrez et al., 1995). Furthermore, it has recently been proposed that the step at which SNAP-25 peptide interferes in the calcium-dependent secretion from permeabilized chromaffin cells is that of vesicle docking (Gutierrez et al., 1997). Taken together, the different experimental approaches shown above which interfered with the normal function of SNAP-25 lead to an inhibition of regulated exocytosis. Other than a critical role in vesicle docking and fusion, SNAP-25 has also been demonstrated to participate in axonal growth. Blocking of SNAP-25 expression by incubating rat cortical neurons and PC12 cells with SNAP-25 antisense oligonucleotides prevents neurite elongation and outgrowth (Osen-Sand et al., 1993).

A Coloboma (Cm) mutant mouse model has been identified, which has helped to access the impact of SNAP-25 on nervous system function. The mutation is a 1-2 cM deletion on mouse chromosome 2 that includes the entire SNAP-25 gene (Snap), but may encompass as many as 30-40 additional linked loci (Hess et al., 1994; Wilson et al., 1996). When homozygous, this gene deletion is embryonically lethal; however, heterozygous mice are viable and exhibit phenotypic abnormalities including ocular dysmorphology, constant head bobbing and profound spontaneous hyperactivity (Searle, 1966; Hess et al., 1994). Cm/+ mice express 50% levels of SNAP-25 mRNA and protein. The involvement of SNAP-25 in the dysregulation of locomotor behaviour resulting from the Cm gene defect was evaluated by genetic complementation with a Snap minigene (Sp). The locomotor activity of Coloboma mutants homozygous for the Sp transgene (genotype Sp/Sp, Cm+/+) was rescued to virtually normal levels. The ability of a Snap transgene to complement the
hyperactivity associated with the Cm gene defects supports the idea that this behavioral abnormality results from a deficiency of SNAP-25 in promoting the vesicle release at synapses (Wilson et al., 1996). Identification of the mouse mutant Coloboma as an animal model for examining the neuro-biochemical basis for hyperactivity will have important implications in understanding hyperactive syndrome such as attention deficit hyperactive disorder (ADHD) which affects 3-5% of school-aged children.

v-SNAREs

VAMP

Vesicle-Associated Membrane Protein (VAMP), also known as synaptobrevin, represents a family of integral membrane proteins of synaptic vesicles that were initially discovered from the electric organ of the marine ray Torpedo californica (Trimble et al., 1988) and subsequently from rat brain (Baumert et al., 1989; Elferink et al., 1989). So far, three mammalian VAMP proteins have been characterized: VAMP-1, VAMP-2 (Elferink et al., 1989) and cellubrevin (McMahon et al., 1993). VAMP-1 consists of 118 amino acids (Mw = 12.760 kDa) and is 84% identical to Torpedo VAMP-1 protein sequence. VAMP-2 is 116-residue in length (Mw = 12.671 kDa) and is 77% and 75% identical to rat and Torpedo VAMP-1 sequences respectively. Cellubrevin contains 103 amino acids and shares 59% identity to both rat VAMP-1 and -2. Three structural domains were found common in VAMPs and cellubrevin (Trimble et al., 1988; Elferink et al., 1989; McMahon et al., 1993; Trimble, 1993). Each member contains an amino-terminal proline rich sequence which is the most divergent region; a well-conserved hydrophilic core which spans about 70 amino acids; and a hydrophobic carboxyl-terminal membrane-spanning region that is composed of 22 residues. Tryptic digestion of intact and lysed synaptic vesicles suggests that the protein is cytoplasmically-oriented (Trimble et al., 1988). Like syntaxin and SNAP-25, all members of the VAMP family exhibit a coiled-coil region
which involves a stretch of 30 residues within the hydrophilic core as illustrated in Fig. B1. The coiled-coil structure is believed to be important for the interaction of SNAP-25 (Chapman et al., 1994) and syntaxin (Calakos et al., 1994) with VAMP.

When VAMP-1 was first isolated and characterized from *Torpedo*, it was found to be expressed only in neuronal tissues such as the brain and purified electric-organ synaptic vesicles (Trimble et al., 1988). Later searches for mammalian counterparts in rat brain identified three related proteins. Synaptobrevin which was isolated from rat brain (Baumert et al., 1989) is identical to VAMP-2 (Sudhof et al., 1989). VAMP-related gene products were also cloned from bovine brain, *Drosophila* (Sudhof et al., 1989; Chin and Goldman, 1992; DiAntonio et al., 1993; Sweeney et al., 1995), human (Archer et al., 1990), squid (Hunt et al., 1994), *Aplysia* (Yamasaki et al., 1994), and yeast (Protopopov et al., 1993). The data on the distribution of VAMP proteins are somewhat inconsistent. In the study which described the cloning of rat VAMP-1 and VAMP-2, both mRNAs were detected only in the central nervous system but not in non-neural tissues examined which included liver, muscle, kidney and heart (Elferink et al., 1989). Subsequent studies on the expression of VAMP-2/synaptobrevin concluded that the protein was detected in neuroendocrine cells and cell lines such as endocrine pancreas, insulinoma cells, adrenal medulla, PC12 cells, but not in other tissues such as muscles and exocrine pancreas (Baumert et al., 1989). However, more recent studies have demonstrated that VAMP/synaptobrevin isoforms 1 and 2 are widely expressed in rat tissues including brain, kidney, heart, and smooth muscle (Rossetto et al., 1996). Moreover, VAMP-2 has been shown to be present in insulin-responsive cells such as adipocytes and skeletal muscle (Cain et al., 1992; Volchuk et al., 1994; Jagadish et al., 1996; Martin et al., 1996; Timmers et al., 1996), as well as secretory cells including neutrophils (Brumell et al., 1995) and exocrine pancreatic acinar cells (Braun et al., 1994; Gaisano et al., 1994). The discrepancy may be due to the methods used for detection and/or the purity of tissue samples. All in all,
it is safe to conclude that VAMP-2 is ubiquitously expressed, cellubrevin may represent a non-neuronal VAMP isoform since it is virtually undetectable in neurons although it is present in glial cells in the brain (McMahon et al., 1993; Chilcote et al., 1995); VAMP-1 distribution may be restricted to neural and endocrine cells.

Several VAMP-related genes were identified in yeast and they include SNC1 (Gerst et al., 1992), SNC2 (Protopopov et al., 1993), SLY1/SEC22 (Dascher et al., 1991; Newman et al., 1992a), BET1 and BOS1 (Newman et al., 1992b; Lian and Ferro-Novick, 1993). Snc1p and Snc2p shared 79% identity to each other and 30-40% identity to mammalian VAMPs. Both of the Snc proteins are constituents of post-Golgi transport vesicles. Bos1p, Bet1p and Sec22p/Sly2p proteins all show limited sequence similarity and share structural features with VAMP. These yeast proteins have been shown to be required for ER to Golgi transport (Dascher et al., 1991; Newman et al., 1992b; Lian and Ferro-Novick, 1993). Summaries of the subcellular localization of VAMPs and the transport step at which each VAMP protein functions are provided in Tables B1 and B2 respectively.

Like SNAP-25 and syntaxin, members of the VAMP family are also cleaved by different Clostridial neurotoxins: BoNT/B, D, F, G and tetanus toxins (Tetx) (reviewed by Niemann et al., 1994). These toxins have been shown to cleave VAMP/synaptobrevin at distinct peptide bonds and therefore become important tools in dissecting the functional importance of VAMPs in vesicle traffic. Injection of tetanus toxin or BoNT/B into Aplysia neurons has been demonstrated to block neurotransmitter release, and the blockade is substantially delayed with peptides containing the synaptobrevin-2 cleavage site (Schiavo et al., 1992). Microinjection of TeTx and BoNT/B light chain into the squid presynaptic terminal effects an irreversible inhibition of neurotransmitter release (Hunt et al., 1994). In adrenal chromaffin cells, inhibition of catecholamine release correlates with cleavage of synaptobrevin and cellubrevin by BoNT/B (Foran et al., 1995). Furthermore, treatment of
permeabilized insulin-secreting cells with TeTx or BoNT/B, which selectively cleave VAMP-2 and cellubrevin, causes an inhibition of Ca\textsuperscript{2+}-triggered insulin exocytosis (Regazzi et al., 1995; Regazzi et al., 1996; Wheeler et al., 1996). In *Drosophila*, genetic mutations removing synaptobrevin have not yet been reported. An alternative approach of expressing the catalytic domain of tetanus toxin in the nervous system of transgenic fly was used to delete the synaptobrevin protein (Sweeney et al., 1995). The loss of synaptobrevin in such transgenic fruitfly completely eliminates synaptic transmission. Moreover, yeast lacking both Snc1p and Snc2p are defective in normal bulk secretion, accumulate large quantity of invertase-containing post-Golgi vesicles, and display a variety of conditional lethal phenotypes which are fully reversible upon the expression of either *SNC1* or *SNC2* from plasmids. Disruption of either *SNC2* or *SNC1* but not both genes was found to be without phenotype, suggesting that the two may play functionally redundant role in post-Golgi transport. Thus, yeast require at least one *SNC* gene to germinate and grow normally (Protopopov et al., 1993).

**Synaptotagmin**

Synaptotagmin I (p65) is an intrinsic membrane protein found on the synaptic vesicles (Matthew et al., 1981) with a domain structure that is conserved in vertebrates and invertebrates (Perin et al., 1991a; Perin et al., 1991b). Nine synaptotagmins are currently known, and they all have the same overall domain structure described below (recently reviewed by Sudhof and Rizo, 1996). All synaptotagmins have a short intravesicular amino terminus which varies in length between 16 to 60 amino acids and is N-glycosylated. This is followed by a transmembrane region (22-25 residues) that contains multiple palmitoylated cysteines (Chapman et al., 1996). Facing the cytoplasm is a stretch of highly charged residues which spans from 37 to 212 amino acids and this is immediately followed by C2A and C2B domains, each of which is comprised of 118-123 amino acids.
The C2A and C2B domains are well-conserved regions that are homologous to the C2 regulatory region found in protein kinase C (Perin et al., 1990). Both C2 domains are stabilized by Ca$^{2+}$, suggesting that they both bind Ca$^{2+}$ (Davletov and Sudhof, 1994). Furthermore, each of the C2A and C2B domains bind two Ca$^{2+}$ ions with intrinsic affinities of 60 µM and of 300-400 µM respectively (Shao et al., 1996; Sudhof and Rizo, 1996). These affinities have been shown to correlate with the Ca$^{2+}$ dependence of exocytosis (Heidelberger et al., 1994), suggesting that synaptotagmin I may function in a Ca$^{2+}$-regulated step of the synaptic vesicle cycle (Perin et al., 1990; Brose et al., 1992).

Native brain synaptotagmin I binds phospholipids in a Ca$^{2+}$-dependent manner (Brose et al., 1992), and this is mediated exclusively by the C2A domain (Davletov and Sudhof, 1994). No binding of phospholipids to the C2 domains of native synaptotagmin I is observed without Ca$^{2+}$ (Li et al., 1995a). Synaptotagmin I has also been shown to bind syntaxin in a Ca$^{2+}$-dependent fashion and the interaction is mediated by the C2A domain of the protein (Li et al., 1995b; Kee and Scheller, 1996). In contrast, β-SNAP and SNAP-25 have been demonstrated to interact directly with the C2B domain of synaptotagmin and these interactions are not dependent on the presence of Ca$^{2+}$ (Schiavo et al., 1995a; Schiavo et al., 1997). Table B3 gives a summary of the molecules that interact with different tagmins.

An often cited evidence questioning the role of SNAREs as docking proteins comes from the observation that synaptic vesicles remain morphologically docked at the presynaptic plasmalemma even after cleavage of SNAREs by Clostridial neurotoxins (Hunt et al., 1994; Goda, 1997). As mentioned previously, synaptotagmin I (v-SNARE) binds, both in vitro and in vivo, to SNAP-25 (t-SNARE) (Schiavo et al., 1997). Furthermore, proteolytic cleavage of SNAP-25 by BoNT/A and BoNT/E does not compromise the binding of SNAP-25 to synaptotagmin. Since synaptotagmin is not cleaved by any of the known neurotoxins, the interaction between synaptotagmin and SNAP-25 could be a
Different regions of the synaptotagmin protein have various degrees of sequence conservation. The intravesicular amino-terminus, the transmembrane region and the highly charged juxtamembrane sequence are conserved between synaptotagmin I and II but varied among other synaptotagmins (reviewed in Sudhof and Rizo, 1996). In contrast, the sequence from C2A to C2B domains is well conserved among all nine synaptotagmins, indicating that the function of C2 domains are conserved among all synaptotagmins. The synaptotagmins not only show varying degree of sequence conservation, they also display distinct pattern of tissue distribution. All synaptotagmins are highly enriched in brain. At least six synaptotagmins are widely expressed in non-neural tissues as well as the brain (III, IV, VI-IX) (Hudson and Birnbaum, 1995; Li et al., 1995a; Sudhof and Rizo, 1996). Synaptotagmin I, II, and III are localized on synaptic vesicles. Synaptotagmin I is also present on large dense core granules and endocrine secretory vesicles (Matthew et al., 1981; Walch-Solimerna et al., 1993; Jacobsson et al., 1994).

Unlike other SNAREs, no known neurotoxins cleave synaptotagmins. However, other biochemical and genetic approaches have been employed to assess the functional importance of this v-SNARE protein. Synaptotagmin I knockout mice were generated and found to develop normally until after birth but die postnatally, suggesting that synaptotagmin I is essential for viability (Geppert et al., 1994). Therefore, in spite of the availability of other eight synaptotagmins, the function of synaptotagmin I is not redundant. Further studies from the hippocampal neurons cultured from these mice revealed that synaptic transmission is severely impaired in the fast phase of Ca\(^{2+}\)-dependent neurotransmitter release. Surprisingly there is no impairment of the slow asynchronous component of neurotransmitter release. Moreover, the frequency of spontaneous miniature release events, the size of the pool of readily releasable synaptic vesicles, and the rate of
replenishment of this pool are also indistinguishable between wild-type and synaptotagmin I knockout mice (Geppert et al., 1994; Sudhof and Rizo, 1996). Therefore these mice suffer from the selective loss of the fast component of Ca^{2+}-dependent exocytosis, without any effect on other synaptic vesicle functions. It was proposed that synaptotagmin I functioned as a Ca^{2+} sensor mediating Ca^{2+} regulation of synchronous neurotransmitter release in hippocampal neurons. Furthermore, direct evidence that synaptotagmin is a Ca^{2+} sensor involved in vesicle fusion comes from Drosophila in which synaptotagmin hypomorphic mutants lacking one of the C2 Ca^{2+} binding domains show a 50% reduction in the Ca^{2+}-dependence of transmission (Littleton et al., 1994). It should be noted that synaptotagmin is by no means the obligatory Ca^{2+} sensor for neurotransmitter release. This is supported by the finding, again from Drosophila, that even though null synaptotagmin mutants are early lethal (DiAntonio et al., 1993; Broadie et al., 1994), Ca^{2+}-dependent neurotransmitter release is still maintained, indicating that other proteins may serve as the Ca^{2+} sensor for release.
Fig. B1 Schematic representation of the predicted coiled-coil domains of SNAREs and α-SNAP which mediate protein-protein interactions (modified from Burgoyne et al., 1996).
Figure B2. Schematic representation of the positions of cleavage of SNAREs by different classical neurexins (modified from Hayashi et al., 1994).
<table>
<thead>
<tr>
<th>Family</th>
<th>Higher eukaryote membrane traffic</th>
<th>Yeast membrane traffic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homolog</td>
<td>Localization</td>
</tr>
<tr>
<td>Syntaxin</td>
<td>t-SNARE</td>
<td>1A, 1B, 2, 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>t-SNARE</td>
<td>SNAP-25</td>
</tr>
<tr>
<td>VAMP/</td>
<td>v-SNARE</td>
<td>VAMP 1, 2</td>
</tr>
<tr>
<td>Synaptobrevin</td>
<td>Soluble</td>
<td>Cellubrevin</td>
</tr>
<tr>
<td>Synaptotagmin</td>
<td>v-SNARE</td>
<td>I - IX</td>
</tr>
<tr>
<td>NSF</td>
<td>Soluble factor</td>
<td>NSF</td>
</tr>
<tr>
<td>SNAP</td>
<td>Soluble factor</td>
<td>α, β, γ</td>
</tr>
</tbody>
</table>

Table B1: Similarity between the subcellular localization of proteins involved in the nerve terminal and yeast transport machinery (see text for details).
Table B2  Similarity between the nerve-terminal and yeast membrane traffic machinery (modified from Bennett and Scheller, 1994).

<table>
<thead>
<tr>
<th>Family</th>
<th>Higher eukaryote membrane traffic</th>
<th>Yeast membrane traffic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homolog</td>
<td>Transport step</td>
<td>Gene</td>
</tr>
<tr>
<td>Syntaxin</td>
<td>t-SNARE</td>
<td>1A, 1B, 2, 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>SNAP-25 t-SNARE</td>
<td>1</td>
</tr>
<tr>
<td>VAMP/Synaptobrevin</td>
<td>v-SNARE</td>
<td>VAMP 1, 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cellubrevin</td>
</tr>
<tr>
<td>Synaptotagmin</td>
<td>v-SNARE</td>
<td>I - IX</td>
</tr>
<tr>
<td>NSF</td>
<td>soluble factor</td>
<td>NSF</td>
</tr>
<tr>
<td>SNAP</td>
<td>soluble factor</td>
<td>α, β, γ</td>
</tr>
</tbody>
</table>
Molecules | Domain of tagmin | Ca\(^{2+}\) dependence | Tagmin isoform studied | Comments |
---|---|---|---|---|
Ca\(^{2+}\) | C2A | 60 \(\mu\)M and 400 \(\mu\)M | I | |

Phospholipids | C2A | I, II, III, V, VII = 5-10 \(\mu\)M IV, VI, VIII, IX = no | All | |

Syntaxin | C2A | I, II, V = 200 \(\mu\)M III, VII = < 1 \(\mu\)M IV, VI, VIII = no | All | |

Synaptotagmin | C2B | 250 \(\mu\)M | I | |

\(\beta\)-SNAP | C2B | No | I | |

SNAP-25 | C2B | No | I | |

IPs | C2B | No | I, II | InsP\(_6\) binds best; InsP\(_4\) & InsP\(_5\) bind; InsP\(_3\) does not bind.
Part II: Models for Vesicle Targeting, Docking, and Fusion

The secretory pathway in yeast, the constitutive protein transport between Golgi cisternae in mammalian cells, and the highly regulated synaptic vesicle exocytosis in mammalian synapses all share a similar protein machinery which underlies a diverse spectrum of intracellular traffic. This strongly suggests that a universal mechanism for vesicle traffic exists in most eukaryotic cells. In Part I, I have introduced the major players of the docking and fusion machinery. In Part II, I will focus on the mechanism by first summarizing a general model for vesicle targeting and docking, known as the SNARE hypothesis, as well as some recent modifications to the hypothesis. The hypothesis was originally proposed by Rothman and colleagues to explain how synaptic vesicles target and dock with the presynaptic plasma membrane. Lastly, I will end this part by introducing an often neglected step in exocytosis, the final membrane fusion event.

Vesicle Targeting and Docking

The SNARE hypothesis: a general vesicle targeting and docking model

As mentioned in Part I, since the specificity of the vesicle traffic machinery cannot be due to NSF and the SNAP proteins, it may be achieved by the specific pairing between SNAREs on the vesicle and the target membrane (Sollner et al., 1993b; Rothman, 1994). Indeed, the interaction among specific SNAREs has become the central postulate of the SNARE hypothesis. Rothman and coworkers proposed that vesicle targeting occurs when v-SNAREs on the donor membrane bind to t-SNAREs on the acceptor membrane. Furthermore, the hypothesis predicts that all eukaryotic cells should have families of v- and t-SNAREs, whose members should form cognate pairs on the basis of binding specificity and should be essential for vesicle docking in vivo (Rothman, 1994).

Based on the SNARE hypothesis as well as experimental data including in vitro protein binding studies, a model for the sequential assembly and disassembly of protein
complexes, shown in Fig. B3, was proposed to correspond to the steps of docking, activation, and fusion of synaptic vesicles (Sollner et al., 1993a; Bennett and Scheller, 1994; Rothman, 1994). In the first step, a complex between two v-SNAREs on the vesicle, VAMP and synaptotagmin, and t-SNAREs on the plasma membrane, syntaxin and SNAP-25, is formed, likely through coiled-coil interactions (Fig. B3, step a). A stable SNARE complex consisting equimolar quantities of syntaxin, SNAP-25, and VAMP has been isolated from detergent extract of brain membranes (Sollner et al., 1993a). In the same report, this SNARE complex was shown to bind to synaptotagmin, which cosediments at 7S in a glycerol gradient. Therefore, the existence of the SNARE complex, either freely or as a synaptotagmin-SNARE complex, implies that a v-SNARE can bind directly to t-SNAREs, which forms a fundamental tenet of the SNARE hypothesis (Sollner et al., 1993a). Synaptotagmin is proposed to serve as a clamp protein which prevents fusion from occurring until a signal is given. In the case of constitutive fusion, the simplest possibility would be that no clamp is present. Indeed, in the constitutive secretory pathway in yeast, no homologue for synaptotagmin has been identified. The synaptic vesicle protein synaptotagmin is believed to be a strong candidate for the role as a fusion clamp for the following reasons: first, it can interact with the SNARE complex; and more importantly, it was reported that endogenous synaptotagmin bound to the native synaptic SNARE complex can be displaced by excess amount of the soluble factor α-SNAP (Sollner et al., 1993a). The potential competition between α-SNAP and synaptotagmin for a common binding site on the SNARE complex suggests a candidate mechanism for negative regulation. When neurotransmitter release is required, the clamp dissociates, possibly in response to increase Ca^{2+} levels, allowing α-SNAP to bind (Fig. B3, step b). The association of α-SNAP with its membrane-bound receptors allow the soluble ATPase, NSF, to bind (Fig. B3, step c). This is consistent with the finding that NSF only binds to SNAPs in the presence of the integral membrane receptors, resulting in the formation of a
multisubunit protein complex with a sedimentation coefficient of 20S when detergent extracts of Golgi membranes containing SNAP receptors are mixed with exogenous NSF and SNAPs in the presence of EDTA (Wilson et al., 1992). Stable 20S particles can also be formed in the presence of either non-hydrolysable analog of ATP, Mg-ATP-γS, or ATP in the absence of magnesium (Wilson et al., 1992; Sollner et al., 1993a). Upon hydrolysis of ATP by NSF, the interactions among NSF, SNAPs and the SNAP receptors are disrupted (Fig. B3, step d). The multisubunit complex has been shown to rapidly dissociate in the presence of Mg-ATP even at 0 °C, liberating NSF in a process that requires ATP hydrolysis (Wilson et al., 1992). The potential conformational changes generated by ATP hydrolysis may either lead directly or indirectly to membrane fusion (Fig. B3, step e). It should be emphasized that this last step is purely a speculation. As Rothman and Wieland indicate in a recent review article, this is the simplest possibility, and it remains unknown whether the physical fusion of bilayers following the NSF ATPase reaction is the direct result of the action of conformationally switched SNAP, SNARE proteins, or both (without the involvement of additional proteins) (Rothman and Wieland, 1996).
Fig. B3 Proposed model for the docking and fusion of synaptic vesicles to the presynaptic plasma membrane at a nerve terminal (from Pevsner and Scheller, 1994c). See text for details.
Two questions have recently prompted the reassessment of the SNARE hypothesis: (1) what is the role of synaptotagmin in docking and fusion of synaptic vesicles? (2) is NSF a fusion protein? Although not directly proven, the bulk of the biochemical and genetic evidence to date indicate synaptotagmin I as part of the Ca\(^{2+}\) sensor for triggering synaptic vesicle fusion (Geppert et al., 1994; Sudhof and Rizo, 1996; Goda, 1997). In order to trigger the final stage of the fusion reaction, synaptotagmin I is required at the site of exocytosis (Sudhof, 1995). According to the SNARE hypothesis, this would be impossible since synaptotagmin is displaced from the core complex upon binding of α-SNAP, long before fusion occurs as shown in Fig. B4, panel (a). In addition, kinetic studies on neurotransmission suggest that the speed of Ca\(^{2+}\)-triggered exocytosis of synaptic vesicles (<200 µs) is too fast for a complex multistep reaction (Sudhof, 1995). Again, the SNARE hypothesis, as shown in Fig. B4, panel (a), proposes that Ca\(^{2+}\) influx occurs prior to the displacement of synaptotagmin by α-SNAP (Rothman, 1994). Moreover, two reports demonstrate that vesicle fusion lags behind Ca\(^{2+}\) influx by only 60 µs at physiological temperatures (Bruns and Jahn, 1995; Sabatini and Regehr, 1996). The exceedingly rapid induction of fusion, therefore, presents a formidable mechanical constraint to the fusion machinery, leaving no time for sequential enzymatic reactions (Goda, 1997), such as the hydrolysis of ATP by NSF.

After docking of synaptic vesicles at the active zone, the vesicles need to go through a maturation process that makes them competent for fast Ca\(^{2+}\)-triggered membrane fusion (Sudhof, 1995). Such a priming step is suggested by two observations: first, most of the docked synaptic vesicles cannot be triggered to fuse by Ca\(^{2+}\) immediately and so they appear as not yet competent for fusion (Hessler et al., 1993; Rosenmund et al., 1993); second, during extensive repetitive stimulation, exocytosis slows down before the number of docked vesicles declines (Wickelgren et al., 1985) indicating that docking is not a rate-
limiting step, rather docked vesicles need to go through a rate-limiting priming step before they are ready to fuse (Sudhof, 1995). Finally, the step at which NSF acts has become highly controversial (Morgan, 1996). According to the SNARE hypothesis, NSF acts as a fusion protein at the post-docking stage close to membrane fusion (Sollner et al., 1993a). However, evidence also exists to suggest a role for NSF at a post-docking but pre-fusion stage (O’Conner et al., 1994). To reconcile these anomalies, an alternative model (Fig. B4 panel (b)) has been suggested (Sudhof, 1995) whereby docking occurs when synaptic vesicle protein, VAMP (v-SNARE), forms a complex with two t-SNAREs on the presynaptic plasma membrane, syntaxin and SNAP-25. This is followed by priming of vesicles when SNAPs and NSF bind to the SNARE docking complex and upon ATP hydrolysis, the core complex is disrupted which leads to hemifusion, possibly by means of the amphipathic N-terminus of VAMP. Synaptotagmin then acts as a Ca^{2+} sensor in completing the fusion reaction by a Ca^{2+}-triggered interaction with syntaxin. Studies have shown that syntaxin binding to synaptotagmin I is regulated by Ca^{2+}, and it requires about 250 μM Ca^{2+} for half-maximal binding (Li et al., 1995b; Kee and Scheller, 1996). This approximates the Ca^{2+} requirement of synaptic vesicle exocytosis (Sudhof, 1995). The post-docking, pre-fusion model for the role of NSF (O’Conner et al., 1994) is consistent with current information on the kinetic steps in exocytosis (Burgoyne and Morgan, 1995), the central theme of which is that Mg^{2+}-ATP-dependent priming occurs before Ca^{2+}-dependent fusion (Morgan and Burgoyne, 1995a). One caveat against the new model is that molecules other than synaptotagmin have to act as a clamp to prevent fusion occurring randomly. Other regulators have indeed been identified and two of them will be discussed in detail in part III.

As Goda pointed out in a recent review article, now that we have gained much information about "who's who" in vesicle traffic for the past several years, the next challenge will be to sort out "who's doing what" in the field of intracellular traffic (Goda,
This would require design of future experiments to establish the minimal set of proteins required for fusion, i.e. to find out which of the protein interactions characterized \textit{in vitro} are physiologically relevant (Goda, 1997), and which ones are "red herrings". Once the minimal set of proteins have been identified, it remains to be investigated how the protein machinery assembly and disassembly are regulated, and more importantly, at what stage of vesicle docking and fusion does it function. One of the major obstacles for addressing whether particular components are required for docking and not in fusion is the difficulty in assessing various states of docked vesicles in synaptic transmission. That is, some docked vesicles may be functionally docked and be fusion competent, whereas others are docked but cannot be fused (Hessler et al., 1993; Rosenmund et al., 1993).
Fig. B4 Two proposed models for synaptic vesicle docking and fusion. (a) A model based on the SNARE hypothesis. (b) An alternative model which differs from the SNARE model in (a) as a result of two modifications: the action of NSF at a post-docking and prefusion stage and the role of synaptotagmin as a Ca$^{2+}$ sensor for vesicle fusion (from Morgan and Burgoyne, 1995a). See text for discussion.
Membrane fusion is ubiquitous throughout biological systems. Membrane fusion is used to transport materials between different intracellular compartments such as the delivery of newly synthesized membrane-bound polypeptides from the endoplasmic reticulum to Golgi, then to their target organelles by constitutive exocytosis. In order to respond to changes in their environment, cells usually undergo regulated exocytosis upon stimulation, for example, transmitter is released to the synaptic cleft upon depolarization of the presynaptic nerve terminal; insulin is secreted by pancreatic β cells in response to high blood sugar. Both constitutive and regulated exocytosis require endoplasmic fusion, in which the cytoplasm-facing leaflets of the membranes fuse (Monck and Fernandez, 1996). Another type of membrane fusion is ectoplasmic, where the fusing bilayer leaflets face the extracellular environment (Monck and Fernandez, 1996). Ectoplasmic fusion occurs during fertilization when the sperm fuses with the egg, and is also used by enveloped viruses to infect cells. Viral ectoplasmic fusion will not be the subject discussed here, but it should be noted that it is unique among all the cellular fusion reactions in that the proteins involved have been identified (White, 1992; Monck and Fernandez, 1996). The extensive studies on viral fusion have been instructive not only in our understanding of the mechanism in ectoplasmic fusion, but also in developing models for endoplasmic fusion which will be the focus here.

Membrane fusion involves the merging of two membranes thereby establishing continuity between the interior of the compartments. The fusion pore has been detected electrically using capacitance measurements in patch-clamped cells (Breckenridge and Almers, 1987; Zimmerberg et al., 1987; Monck and Fernandez, 1996). These studies, mainly using mast cells to study secretory granule fusion with the plasma membrane, have provided the following picture of the exocytotic fusion pore (Monck and Fernandez, 1996): (1) the fusion pore is observed as an abrupt (<1 ms) appearance of a small electrical
conductance, which is equivalent to a pore size of 1-2 nm in diameter; (2) the pore undergoes rapid fluctuations (flicker) in conductance without any distinct, stable conductance levels; (3) during this flicker phase there is a lipid flux through the pore, suggesting that the secretory granule membrane is under tension and that the dynamic behavior of the pore might be explained by a changing lipid composition; (4) the flickering of the fusion pore usually culminates in irreversible expansion of the pore to >10 nm diameter and release of the secretory granule contents; (5) the fusion pore sometimes closes, leaving an intact secretory granule inside the cell; (6) the closure of the fusion pore has an unusual, discontinuous temperature dependence, indicative of a process that depends on lipid composition. Two main conclusions were derived from this sequence of observations: first, the fusion pore is a predominantly lipid structure; second, the fusion-pore formation occurs after hemifusion.

The freeze-fracture electron microscopy also revealed a likely precursor of membrane fusion (Monck and Fernandez, 1996). Following mast cell stimulation, the plasma membrane invaginated in the form of a highly curved cusp or 'dimple', which spanned the 100 nm gap between secretory vesicles and the plasma membrane (Chandler and Heuser, 1980). Similar dimples have been seen in other cells, although the sizes were variable (Ornberg and Reese, 1981; Schmidt et al., 1983), with a separation of only 5-20 nm at the presynaptic terminal of the neuromuscular junction (Heuser and Reese, 1981). Similarly, by using atomic force microscopy, a recent report described the images of 'pits' measuring 500-2000 nm in diameter, each of which contains 3-20 depressions measuring 100-180 nm in diameter, observed at the apical surface of living pancreatic acinar cells (Schneider et al., 1997). Furthermore, the depressions widened to about 200 nm during amylase secretion and returned to their control size upon cessation of the secretory response. Changes were observed only in the depressions upon stimulation of secretion. The authors proposed that the depressions correspond to the sites where the exocytotic
fusion pores form during amylase secretion. The mast cell dimples measured about 100 nm at their mouths (Chandler and Heuser, 1980), and are, thus similar to the size of the depressions measured in the acinar cells (Schneider et al., 1997). However, in mast cells, the dimples were observed only upon stimulation whereas the depressions of the pancreatic acinar cells observed by atomic force microscopy were detected at resting state (Fernandez, 1997). Fernandez, in his commentary, suggests that it is possible that part of what is being imaged by atomic force microscopy is the hard shell of the underlying cytoskeleton, therefore the images may be more complex than simple topographic representation of fusion pores (Fernandez, 1997).

Observations from some freeze-fracture electron micrographs suggest that a macromolecular scaffold of proteins brings the plasma membrane into close proximity with the secretory granule (Monck and Fernandez, 1992; Nanavati et al., 1992; Oberhauser and Fernandez, 1993). These results led to a scaffold model for exocytotic fusion (Monck and Fernandez, 1992; Nanavati et al., 1992), in which the function of the proteins in the scaffold is to draw the plasma membrane into the dimple shape, probably as a result of an interaction of the scaffold proteins with the head groups of the lipids (Monck and Fernandez, 1994; Monck and Fernandez, 1996). For ectoplasmic viral fusion, the scaffold is composed of hemagglutinin proteins or other viral proteins (Bentz et al., 1990; Hoekstra, 1990; White, 1992; Zimmerberg et al., 1993). It has been suggested that the SNARE proteins, NSF and SNAPs play a role in targeting the fusion reaction, but as yet, the proteins involved in promoting the fusion reaction remain unknown.

According to the exocytotic scaffold model (Monck and Fernandez, 1996) shown in Fig. B5, the sequence of events leading to the final membrane fusion are as follows: (i) the membranes are separated by a protein scaffold of unknown identity; (ii) in response to the appropriate stimulus (which may involve a rise in Ca$^{2+}$ or presence of GTP-binding proteins), the scaffold directs a dimple in the plasma membrane towards the secretory
granule; (iii) hemifusion of the apposing leaflets occurs spontaneously due to attractive forces that are exposed by the membrane curvature and local tensions in the membranes; (iv) a small pore forms in the stressed common bilayer of the hemifusion structure; at this stage, the pore can either close or expand; (v) the fusion pore develops into the hour-glass structure, spanning both membranes; (vi) the pore further expands to allow release of secretory products. An important observation provided by patch-clamp measurements of mast cells was that a fusion pore does not always expand irreversibly but sometimes closes, leaving an intact secretory vesicle inside the cell (Fernandez et al., 1984). Transient fusion events have since been reported in other cell types including neutrophils (Lollike et al., 1995) and adrenal chromaffin cells (Robinson et al., 1996). Likewise, some release events observed in neurons may also correspond to release through a transient fusion pore (Monck and Fernandez, 1996; Robinson et al., 1996), which allows discharge of the content of vesicle and then close, obviating the need for membrane merging and subsequent recycling (Valtorta et al., 1990).

The hemifusion step in the scaffold model (Fig. B5, stage iii) is consistent with the alternative model to the SNARE hypothesis mentioned above. According to the modified SNARE hypothesis, the hydrolysis of ATP by NSF results in a disruption of the core complex which leads to hemifusion, possibly by means of the amphipathic N-terminus of VAMP (Sudhof, 1995). A possible candidate as a scaffold protein is synaptotagmin I since it can form homo-multimers and the polymerization reaction has a Ca$^{2+}$ dependence (about 250 μM) (Chapman et al., 1996; Sugita et al., 1996) which approximates the Ca$^{2+}$ requirement of synaptic vesicle exocytosis (Sudhof, 1995). For viral fusion, where the scaffold is composed of hemagglutinin proteins, the hemagglutinin proteins polymerize as trimers and the trimers assemble to form a 'collar' within which the fusion pore forms (Bentz et al., 1990; White, 1992; Danieli et al., 1996). The Ca$^{2+}$-triggered polymerization of synaptotagmin I mirrors the low-pH induced activation of the viral hemagglutinin
proteins (Weber et al., 1994). This suggests synaptotagmin I as a likely scaffold molecule for exocytotic fusion, similar to the way in which polymerization of hemagglutinin proteins causes viral fusion.
Fig. B5 Scaffold model for membrane fusion during exocytosis (from Monck and Fernandez, 1996). See text for description of each step.
An often asked question regarding the theme of universality proposed by the SNARE hypothesis is the following: how can constitutive fusion machinery also be used in triggered exocytosis? The simplest possibility is that in regulated exocytosis, a molecule can act as a clamp which prevents the formation of the 20S fusion particle. Upon stimulation, the clamp will be released so that SNAPs and NSF can associate with the SNARE complex leading to membrane fusion. Synaptotagmin I has been proposed as a fusion clamp since it can associate with the SNARE complex (Sollner et al., 1993a); moreover, it competes with α-SNAP for the binding to a common site on the SNARE complex (Sollner et al., 1993a), hence a candidate mechanism for regulation of the exocytotic process. Here, I will introduce two additional proteins which can serve as potential regulators of vesicle traffic.

\[(1) \textit{n-Sec1 / Munc18 / rbSec1}\]

\textit{SEC1} gene was isolated as one of the ten essential late-acting secretory (sec) mutants that are involved in the transport between ER and the plasma membrane in yeast (Novick et al., 1981). Sly1p is a homologue of Sec1p and is essential for transport between endoplasmic reticulum and the Golgi in the yeast secretory pathway (Pelham, 1993; Sogaard et al., 1994). Unc-18 (Hosono et al., 1992; Gengyo-Ando et al., 1993) and Rop (Ras opposite protein) (Harrison et al., 1994) are Sec1p homologues in \textit{Caenorhabditis elegans} and \textit{Drosophila}, respectively. The neural homologue of Sec1p in higher animals are referred to as n-Sec1 (Pevsner et al., 1994b), Munc-18 (Hata et al., 1993), or rbSec-1 (Garcia et al., 1994).

An Unc-18 mutant has been shown to inhibit transmitter release from the presynaptic nerve terminals (Hosono et al., 1992). Similarly, the Rop null mutant flies die at late embryonic stages and show numerous pleiotropic defects consistent with a loss of
exocytosis in a number of neuronal and non-neuronal cell types (Harrison et al., 1994). Moreover, viable temperature-sensitive mutations of Rop show loss of synaptic activity in response to light stimulus, suggesting that the protein is required for synaptic transmission (Harrison et al., 1994). Interestingly, overexpression of Rop protein likewise suppresses synaptic transmission at the neuromuscular junctions (Schulze et al., 1994). These genetic results suggest a negative regulatory role for vertebrate n-Sec1 in synaptic vesicle release (Pevsner et al., 1994b). n-Sec1 binds to syntaxins 1, 2, and 3, but not 4 (Pevsner et al., 1994b), and syntaxin but not SNAP-25 or VAMP binds to n-Sec1 (Pevsner et al., 1994a). Furthermore, in the same report, n-Sec1 was shown to be absent from both the 7S and 20S particles, indicating that syntaxin can exists in at least three different states: as part of either 7S or 20S particles, or with n-Sec1. These were proposed to represent three different intermediates in the vesicle docking and fusion pathway (Pevsner et al., 1994a). Two alternatively spliced variants of Munc-18, Munc-18b and Munc-18c, were identified in adipocytes (Tellam et al., 1995). Munc-18b, like n-Sec1, only binds to syntaxin 1A, 2 and 3 but not 4, while Munc-18c binds only to syntaxins 2 and 4 (Tellam et al., 1997). Using a three-way binding assay, it was shown that Munc-18c, like n-Sec1, inhibits the binding of t- and v-SNAREs, in this case, syntaxin 4 and VAMP-2 (Tellam et al., 1997).

The finding that n-Sec1 inhibits binding of either VAMP or SNAP-25 to syntaxin leads to the development of a working model in which n-Sec1 associates with syntaxin and may regulate vesicle docking by preventing SNAP-25 and VAMP binding to syntaxin. As the vesicle docks, another unknown factor could release n-Sec1 from syntaxin, which is followed by the formation of the 7S complex (Pevsner et al., 1994a) (Fig. B3). The high affinity of n-Sec1 for syntaxin (binding constant in nanomolar range), which is 1000-fold greater than that of VAMP to syntaxin, suggests that the transition of syntaxin from an n-Sec1-bound state to a vesicle-bound state is highly regulated (Pevsner et al., 1994a).
A recent study demonstrated that n-Sec1 can be phosphorylated at two Ser residues by protein kinase C (Fujita et al., 1996). Phosphorylation prevents interaction of n-Sec1 with syntaxin 1A, and n-Sec1 proteins that are already bound to syntaxin 1A cannot be phosphorylated. These results imply that phosphorylation of n-Sec1 may block its re-interaction with syntaxin, after it dissociates from syntaxin 1A (Fujita et al., 1996); however, phosphorylation cannot be the mechanism to release n-Sec1 from syntaxin since bound n-Sec1 cannot be phosphorylated. It remains to be determined how the interaction between n-Sec1 and syntaxin is regulated.

(2) Rab

Rab represents a family of more than 30 members of low molecular mass GTP-binding proteins in mammals. All Rab proteins are intrinsically hydrophilic, but are attached to membranes via C-terminal isoprenoid (geranyl-geranyl) and fatty acid chains (Rothman, 1994). Individual members of the Rab family localize to specific subcellular compartments and are implicated at essentially all steps of membrane transport that have been studied (for recent reviews, see Pfeffer, 1992; Ferro-Novick and Novick, 1993; Zerial and Stenmark, 1993). For example, in the endocytic pathway, early and late endosomes each contain a unique set of Rab proteins, Rab4 and Rab5 on early endosomes and Rab7 and Rab9 on late endosomes (Gruenberg and Maxfield, 1995). Synaptic vesicles contain at least two Rab proteins: Rab3A and 3C, which are specific for synaptic vesicles and secretory granules (Fischer von Mollard et al., 1994). Rab3A is the most abundant Rab protein in the brain, accounting for approximately 25% of the Rab GTP-binding capacity of this tissue (Geppert et al., 1994). Most neurons express Rab3A, and a subpopulation of neurons also synthesize high levels of Rab3C (Li et al., 1994). Rab homologues have also been identified in yeast: Ypt1p controls the endoplasmic reticulum
to Golgi transport (Segev et al., 1988; Segev, 1991), whereas Sec4p is involved in the transport between Golgi and the plasma membrane (Goud et al., 1988).

The findings that many of the Rab proteins are localized to specific intracellular membrane compartments and are implicated in intracellular membrane regulation led to the suggestion that they might provide an additional level of targeting specificity possibly through interactions with the SNAREs (Sogaard et al., 1994). In the mammalian system, the only evidence which suggests that Rab proteins may associate with the SNARE complex comes from the finding that using an antibody which recognizes both SNAP-25 and Rab 3A, a complex containing syntaxin 1 and VAMP, which were detected by Coomassie blue staining, was precipitated from bovine brain extracts (Ishizuka et al., 1995). This observation could result from one of two situations: either the Rab 3A indeed forms a complex with the SNAREs or that SNAP-25, but not Rab 3A, is responsible for coprecipitating syntaxin 1 and VAMP. Therefore the apparent detection of Rab 3A as part of the SNARE complex is due to a lack of specificity of the antibody. Using an antibody that recognizes specifically syntaxin 1A and 1B, Rab 3A was not found in the coprecipitate, suggesting that the latter explanation was probably true and Rab 3A is not part of the SNARE complex. In yeast, interactions between Rab homologues and the SNAREs have been well described by genetic and biochemical approaches and they are summarized below (Henry et al., 1996).

Although both yeast Rab proteins, Sec4p and Ypt1p are essential gene products for transport in yeast, a number of proteins have been identified which when overexpressed can compensate for the loss of Rab function in yeast (Dascher et al., 1991; Brennwald et al., 1994). Overexpression of SEC9, a yeast gene encoding a homologue of the 25 kDa synaptosomal-associated protein (SNAP-25), was found to suppress a mutation in the effector domain of Sec4p, which is a Golgi-to-plasma membrane Rab protein (Brennwald et al., 1994). Since overexpression of downstream components can often suppress defects
in upstream gene products (Kim, 1994), given the nature of the Sec4p defect and the nature of the suppression, these results are consistent with the idea that Sec9p acts downstream of Sec4p in the exocytic pathway (Brennwald et al., 1994), although the results do not exclude the possibility that Sec4p and Sec9p represent independent parallel pathways which lead to the same effect. In the same study, the investigators could not cross-link or coimmunoprecipitate Sec4p with Sec9p, indicating that Sec4p is not part of the corresponding SNARE complex; therefore, regulation of SNAREs by Rabs may require the action of intermediate components (Brennwald et al., 1994).

A mutation affecting the function of Ypt1p, which controls the endoplasmic-reticulum-to-Golgi transport, inhibited the assembly of the corresponding SNARE complex (Lian et al., 1994; Sogaard et al., 1994). Temperature-sensitive mutants of Ypt1p were identified, and inactivation of Ypt1p by shifting to restrictive temperature prevented the accumulation of the SNARE-containing complex of Bos1p (v-SNARE of yeast endoplasmic reticulum-to-Golgi transport vesicles) and Sed5p (t-SNARE of endoplasmic-reticulum-to-Golgi transport) (Sogaard et al., 1994). These results clearly imply that Ypt1p function is required for the assembly of the endoplasmic reticulum-Golgi SNARE complex. Since Ypt1p protein is not itself found in the SNARE complex, Ypt1p is not likely to contribute a core interaction necessary for the stability of this docking complex (Sogaard et al., 1994). Though important, the requirement for Ypt1p can nonetheless be by-passed by overexpressing certain v-SNAREs (Dascher et al., 1991). In the absence of functional Ypt1p, the two endoplasmic reticulum-Golgi v-SNAREs Bos1p and Sec22p fail to form a complex (Lian et al., 1994), hence, functional Ypt1p promotes the formation of the Bos1p-Sec22p complex. Moreover, disruption of YPT1 was efficiently by-passed when SEC22 was co-overexpressed with BOS1. The investigators hypothesize that Sec22p aids the activity of Bos1p on vesicles, allowing Bos1p to interact more efficiently with its receptor or t-SNARE (proposed to be Sed5p). Since Ypt1p does not directly interact with Bos1p
and See22p, Rabs may regulate the specificity of vesicular transport by indirectly promoting the oligomerization of the v-SNAREs on transport vesicles (Lian et al., 1994).

Taken together, these observations suggested that Rab action must somehow facilitate the assembly of the SNAREs. Because they form no part of the isolated docking complex containing SNARE proteins, Rab proteins may therefore catalyze unique v- and t-SNARE interactions (Rothman, 1994), thereby contributing an additional layer of targeting specificity. Although highly suggestive, these studies have not yet demonstrated a direct role for Rab proteins in SNARE complex formation. It remains to be revealed the stage at which Rab proteins function during vesicle docking and fusion.
Part IV: Role of SNAREs in Insulin-Stimulated Glucose Transport

Glucose is the main energy source for most mammalian cells. In the fasting state, glucose is released into the circulation from the liver as a result of the breakdown of glycogen. After a meal, the blood sugar level rises, stimulating the pancreatic β cells to release insulin into the circulation. The post-prandial clearance of glucose from the circulation is tightly regulated by insulin, which clears blood glucose in two ways: first, to prevent the liver from releasing more glucose; second, to cause hepatocytes, muscle and fat cells to absorb glucose from the circulation. In hepatocytes and muscle cells, glucose is stored as glycogen, whereas in adipocytes, glucose is converted to triglycerides for long-term storage. As the blood glucose level drops, the pancreatic β cells stop secreting insulin, and the body's metabolism returns to basal state.

Since the lipid bilayer of the plasma membrane is impermeable to glucose, the uptake by muscle and fat cells must be facilitated by specific transporter proteins called GLUTs (GLUCose Transporters). A family of six facilitative glucose transporters (GLUTs 1-5, and 7) has been characterized (GLUT6 is a pseudogene), which transport glucose down its chemical gradient. The GLUT family is characterized by single polypeptides of about 500 amino acids in length, each of which has 12 predicted membrane-spanning helices (reviewed by Gould and Holman, 1993; James, 1995; Taha, 1996; Klip and Marette, 1997). The GLUTs are expressed in a tissue-specific manner. Expression of GLUT4, for example, is confined to cell types that exhibit insulin-stimulated glucose transport, such as adipose tissue, skeletal and cardiac muscles (James et al., 1988; Birnbaum, 1989; James et al., 1989; James, 1995). In contrast to the tissue-specificity of GLUT4, GLUT1 is expressed in many cells throughout the body, including the insulin-responsive tissues, but at much higher levels in endothelial cells within the brain, placenta, eye and testis (James, 1995).
Kinetic studies of glucose uptake into fat and muscle suggest that insulin increases the $V_{max}$ of glucose transport (Birnbaum, 1989; Gould and Holman, 1993; Stephens and Pilch, 1995). In 1980, two independent studies by Cushman and Wardzala, and Suzuki and Kono, first demonstrated an increase in glucose transporter number at the cell surface in response to insulin. Glucose transporter distribution was assessed in plasma membrane fraction and microsomes of rat fat cells that were isolated before and after exposure to insulin (procedure described by Simpson et al., 1983). Cushman and Wardzala used a labeled fungal metabolite, cytochalasin B, which binds directly to glucose transporters, to assess the number of transporters in the membrane fractions (Cushman and Wardzala, 1980). Suzuki and Kono measured glucose uptake in isolated membrane fractions from rat adipose cells (Suzuki and Kono, 1980). Both groups reached the same conclusion, that in unstimulated adipose cells most of the glucose transporters (now known to be predominantly GLUT4) are associated with an intracellular (microsomal) membrane compartment. Upon exposure to insulin, the transporters are 'recruited' from an intracellular site to the cell surface. These results led to the transporter recruitment hypothesis, which postulates that an increase in the number of functional transporters on the surface is the major mechanism for insulin stimulation of glucose transport in both fat and muscle tissues (Stephens and Pilch, 1995). Therefore, when the insulin level is low, the hypothesis predicts that most of the glucose transporters are sequestered intracellularly. When the level of insulin is high, these transporters are mobilized from the intracellular compartment to the cell surface to mediate the influx of glucose.

The cloning and characterization of different isoforms of glucose transporters as well as the availability of isoform-specific antibodies, allow re-examination of the insulin-stimulated glucose uptake by rat adipocytes. Immunoblotting and photolabeling studies showed that native rat adipocytes express glucose transporter proteins, GLUT1 and GLUT4. The level of GLUT1 is about 5-10% that of GLUT4 (Oka et al., 1988; Zorzano...
due to its much higher abundance, GLUT4 is by far the predominant species responsible for the 20-30-fold increase in glucose transport as a result of exposure of fat cells to insulin (Whitesell and Gliemann, 1979; Taylor and Holman, 1981; May and Mikulecky, 1982; Simpson and Cushman, 1986; Holman et al., 1990). In unstimulated adipocytes, more than 95% of GLUT4 is located intracellularly (Rice et al., 1996) whereas more than 50% of GLUT1 resides at the plasma membrane, with the rest being sequestered inside adipocytes (Kandror et al., 1995). The ubiquitous distribution of GLUT1 in many tissues and its preferential localization at the cell surface in unstimulated rat adipocytes suggests its function in maintaining the growth and metabolism of a cell. GLUT4, on the other hand, is expressed only in insulin-responsive cells and the majority of GLUT4 is sequestered intracellularly in unstimulated cells. These two characteristics of the distribution of GLUT4 together with the observations that insulin causes an increase in the cell surface transporter number (Cushman and Wardzala, 1980; Suzuki and Kono, 1980) strongly suggest that insulin-stimulated glucose influx is mediated predominantly, if not entirely, by the translocation of GLUT4 to the plasma membrane. It should be noted that the intracellular GLUT1 pool, although less significant, is also translocated to the cell surface after insulin administration, just like the GLUT4-containing vesicles (Zorzano et al., 1989; Kandror et al., 1995; Kandror and Pilch, 1996).

Skeletal muscle is the major site for whole body insulin-stimulated glucose disposal, whereas adipocytes contribute to a relatively minor degree in normal individuals (Kraegen et al., 1985); therefore, effort has been made to characterize the mechanism of the insulin-dependent glucose transport activation in this tissue. Despite considerable technical difficulties in the fractionation of skeletal muscle, it has been shown that the major molecular mechanism of insulin action, namely the recruitment of GLUT4 protein to the
The mechanism by which insulin stimulates glucose uptake by fat and muscle has been studied vigorously due to its clinical relevance in diabetes mellitus. There are two major categories of the disease: Insulin-Dependent Diabetes Mellitus (IDDM) and Non-Insulin-Dependent Diabetes Mellitus (NIDDM). The majority of diabetic patients suffer from NIDDM. It has been shown that NIDDM patients have a normal complement of GLUT4, at least in muscle, which is the major target tissue for insulin-stimulated glucose disposal (Kahn, 1992; Livingstone et al., 1996). Therefore defects in translocation of GLUT4 to the cell surface in response to insulin may underlie the insulin resistance observed in these patients (Rice et al., 1996). There are two phenomena in the translocation of GLUTs by insulin: first, the signalling pathway responsible for activating the mobilization of the intracellularly-sequestered glucose transporter; second, the targeting and docking of GLUT4-containing vesicles with the plasma membrane in response to insulin.

The insulin signalling pathway starts when insulin binds to its receptor on the cell surface. The insulin receptor is composed of two extracellular α-subunits that are linked to two membrane-spanning β-subunits by disulfide bonds (reviewed by White and Khan, 1994; Taha, 1996). The binding of insulin to the α-subunit causes an auto-phosphorylation of the β-subunit on tyrosine residues, which leads to an activation of the tyrosine kinase of the β-subunit. This is followed by phosphorylation of several substrates on tyrosine residues, of which IRS-1 (Insulin Receptor Substrate-1) is the best characterized. IRS-1 is a cytoplasmic protein with multiple tyrosine phosphorylation sites that, following insulin stimulation, serves as docking sites for intracellular molecules that contain specific recognition domains, termed SH2 (Src Homology 2) domains (Kahn, 1995). Protein-protein interactions mediated by specific phosphotyrosines and SH2
domains form the basis of many of the interactions in the signaling pathway (White and Khan, 1994; Seaman et al., 1996). A strong SH2-domain-containing candidate protein directly involved in the GLUT4 translocation pathway is the enzyme Phosphatidylinositol 3-kinase (PI 3-kinase). Pharmacological agents such as the fungal metabolite, wortmannin, and a synthetic inhibitor of PI 3-kinase, LY 294002, which effectively abolish the activity of the enzyme, also potently block the translocation of GLUT1 and GLUT4 to the plasma membrane (Cheatham et al., 1994; Clarke et al., 1994; Tsakiridis et al., 1995), and glucose transport (Cheatham et al., 1994; Okada et al., 1994; Tsakiridis et al., 1995) upon exposure of insulin to muscle and fat cells. PI 3-kinase contains a regulatory subunit of 85 kDa (p85) whose two SH2 domains interact with the phosphotyrosines of IRS-1, as well as a 110-kDa catalytic subunit (p110) which phosphorylates inositol lipids on the D-3 position, converting Phosphatidylinositol (PI), PI-4-monophosphate and PI-4,5-bisphosphate to PI-3-monophosphate, PI-3,4-bisphosphate and PI-3,4,5-trisphosphate respectively (for recent review, see Taha, 1996; Klip and Marette, 1997). The physiological significance of the production of D-3-phosphorylated phosphoinositides remains to be determined.

Although necessary, the activity of PI 3-kinase is not sufficient to stimulate GLUT4 translocation in response to insulin. This is suggested by the observation that other growth factors such as Platelet-Derived Growth Factor (PDGF) and interleukin 4, which are potent activators of PI 3-kinase, do not stimulate glucose transport (Isakoff et al., 1995; Sale et al., 1995). Since insulin promotes the association of activated PI 3-kinase with intracellular membranes, whereas PDGF causes association of the kinase with the plasma membrane (Nave et al., 1996; Ricort et al., 1996), it was proposed that the unique localization of PI 3-kinase activated by insulin in part determines its effect on the translocation of glucose transporters (Klip and Marette, 1997). This hypothesis is supported by a recent finding that after a short exposure of insulin to fat cells,
immunoisolated intracellular GLUT4 vesicles contain PI 3-kinase-IRS-1 complex (Heller-Harrison et al., 1996). From this observation, it is speculated that arrival of PI 3-kinase to the GLUT4-containing vesicles primes them for translocation (Klip and Marette, 1997). Future experiments are needed to address the consequences after priming of GLUT4 vesicles. Ultimately, the insulin-signalling pathway leads to the mobilization of the GLUT4 proteins from an intracellular compartment to the cell surface.

The second site of regulation in the process of transporter recruitment is the targeting and docking of GLUT4-containing vesicles with the plasma membrane. The SNARE hypothesis, which predicts that all eukaryotic cells should have families of v- and t-SNAREs, to form cognate pairs on the basis of binding specificity (Rothman, 1994), allows easy testing for the relevance of the model in insulin-regulated GLUT4 traffic. Several SNARE proteins have since been identified in muscle and fat cells after the initial proposal of the SNARE hypothesis, and they are summarized below.

**v-SNAREs**

**VAMPs**

In an attempt to search for similarities between the insulin-regulated exocytosis of GLUT4 and transmitter release at synapses, Cain et al. screened the GLUT4-containing vesicles isolated from rat adipocytes for proteins that were found on synaptic vesicles by immunoblotting (Cain et al., 1992). Two proteins immunologically related to VAMPs were found. In response to insulin, the amount of VAMPs in the intracellular membrane compartment reduced with a concomitant increase in the plasma membrane. The same effect was observed on the distribution of GLUT4 protein in response to insulin (Cain et al., 1992). Subsequent studies have confirmed that the two VAMP-related immunoreactive species are VAMP-2 and cellubrevin/VAMP-3 that are present on the GLUT4 vesicles (Volchuk et al., 1995; Tamori et al., 1996), and in the intracellular membrane fraction of
3T3-L1 adipocytes (Jagadish et al., 1996; Martin et al., 1996) and rat adipocytes (Timmers et al., 1996). VAMP-2 and cellubrevin have also been reported to be present in rat skeletal muscle and cultured L6 myotubes, and they are found to be preferentially enriched in the intracellular membrane compartment (Volchuk et al., 1994). The functional importance of the VAMPs in insulin-regulated GLUT4 exocytosis is assessed by incubating permeabilized 3T3-L1 adipocytes with BoNT/D (Cheatham et al., 1996) or BoNT/B (Tamori et al., 1996) that cleave VAMP-2 and cellubrevin. Cleavage results in an inhibition of insulin-stimulated translocation of GLUT4-containing vesicles to the plasma membrane and of glucose uptake (Cheatham et al., 1996; Tamori et al., 1996). Furthermore, incubation of permeabilized adipocytes with recombinant soluble VAMP-2 protein, which interferes with the association of native membrane-bound VAMP-2 with other SNARE proteins that are necessary for vesicle docking to occur, also effectively blocks the insulin-dependent translocation of GLUT4 to the cell surface (Cheatham et al., 1996).

**Synaptotagmins**

Several synaptotagmin isoforms (tagmins) are expressed ubiquitously in different tissues, of which synaptotagmin IX (originally reported as synaptotagmin-5) was identified as a nonneuronal isoform by screening a rat adipose tissue library with a synaptotagmin I cDNA probe (Hudson and Birnbaum, 1995). Synaptotagmin IX mRNA was detected in brain, adipose and cardiac tissues. In neurons, synaptotagmin I serves as a Ca$^{2+}$ sensor for neurotransmitter release (Sudhof and Rizo, 1996). Although there was no direct information on the Ca$^{2+}$-binding properties of synaptotagmin IX, it was shown that, unlike synaptotagmin I which demonstrates Ca$^{2+}$-dependent binding of phospholipids, synaptotagmin IX lacks this property (Hudson and Birnbaum, 1995). In muscle and fat cells, it is generally believed that Ca$^{2+}$ does not play a significant role in insulin-stimulated
recruitment of glucose transporters, therefore synaptotagmin IX is unlikely to act as a Ca sensor in these cells. The investigators could not characterize the subcellular localization of synaptotagmin IX, which would be important in determining its role in GLUT4 translocation, due to its low level of expression and the insensitivity of antisera (Hudson and Birnbaum, 1995). Furthermore synaptotagmin IX mRNA was found in adipose and cardiac tissues, but not in skeletal muscle, suggesting that either skeletal muscle expresses an additional isoform of synaptotagmin, or synaptotagmin IX is unlikely to be important in insulin-regulated glucose transport (Hudson and Birnbaum, 1995).

**t-SNAREs**

**Syntaxins**

Two isoforms of syntaxin, syntaxins 2 and 4, have been detected in 3T3-L1 adipocytes (Jagadish et al., 1996; Volchuk et al., 1996; Tellam et al., 1997), rat adipose cells (Timmers et al., 1996), rat skeletal muscle and cultured L6 muscle cells (Sumitani et al., 1995). Both syntaxins 2 and 4 are enriched in the plasma membrane fraction. Insulin causes intracellular syntaxin 4 (Volchuk et al., 1996; Tellam et al., 1997), but not syntaxin 2 (Volchuk et al., 1996) to redistribute to the plasma membrane similar to GLUT4. The presence of syntaxins which are supposedly t-SNARE molecules in the intracellular compartment suggest that the proteins are involved in traffic other than insulin-stimulated GLUT4 exocytosis. Introducing anti-syntaxin 4 antibodies to 3T3-L1 adipocytes by either using permeabilized cells or microinjecting into intact cells results in a significant reduction in insulin-dependent stimulation of glucose transport and recruitment of GLUT4 proteins to the cell surface (Volchuk et al., 1996; Tellam et al., 1997). These results clearly indicate a role of syntaxin 4 in the recruitment of GLUT4 and insulin-dependent stimulation of glucose uptake.
The identification of two members of Munc-18 family, Munc-18b and c, in 3T3-1E adipocytes (Tellam et al., 1995) suggests a role for these cytosolic proteins in modulating the accessibility of syntaxins. Munc-18b, like the neuronal Munc-18/nsec1, binds to syntaxins 1A, 2, and 3, while Munc18c binds to syntaxins 2 and 4 (Tellam et al., 1997). The interaction between Munc-18c and syntaxin 4 is of particular interest since syntaxin 4, other than the neural-specific syntaxin 1, is the only syntaxin that interacts with VAMP-2 (Calakos et al., 1994). Using a three-way binding assay, it was shown that Munc-18c inhibited the interaction between syntaxin 4 and VAMP-2 (Tellam et al., 1997). It is tempting to propose that in an unstimulated cell, the majority of syntaxin 4 is associated with Munc-18c; upon insulin stimulation, Munc-18c dissociates from syntaxin 4 thereby allowing VAMP-2 on the GLUT4 vesicles to bind to syntaxin 4 on the plasma membrane. Future studies will have to validate this hypothesis.

SNAP-25

Unlike the SNARE proteins mentioned above where isoforms are found in non-neuronal tissues, the expression of SNAP-25 is restricted to neural and neuro-endocrine cells only. According to the SNARE hypothesis, functional equivalents of SNAP-25 should exist outside the nervous system to allow intracellular vesicle traffic to occur in non-neural tissues. The search for non-neural equivalent of SNAP-25 will be the focus of the remainder of this Thesis.
The SNARE hypothesis has been proposed as a universal docking and fusion mechanism for intracellular traffic in eukaryotic cells. The model postulates that vesicle targeting occurs when vesicle-associated membrane protein, VAMP/synaptobrevin (v-SNARE), binds to two proteins on the target membrane, synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin (t-SNAREs). According to the SNARE hypothesis, eukaryotic cells should have families of v- and t-SNAREs whose members form cognate pairs that define the targeting specificity. Indeed, homologues of these proteins have been identified, some of which are neural-specific, such as VAMP-1 and syntaxin 1, whereas some are ubiquitously expressed. The expression of the two alternatively spliced variants of SNAP-25, SNAP-25a and SNAP-25b, is restricted to neural and neuro-endocrine cells only. According to the SNARE hypothesis, functional equivalent of SNAP-25 must exist outside the nervous system to allow vesicle traffic in non-neural tissues. Therefore the aim of this Thesis is to search for a non-neural equivalent of SNAP-25, as well as to examine its tissue distribution and subcellular distribution in non-neural cells.
INTRODUCTION

As discussed in previous sections, the SNARE hypothesis of vesicle traffic predicts that vesicle targeting occurs when v-SNAREs on the vesicle and t-SNAREs on the target membrane form a complex which is necessary for vesicle fusion. Moreover, the model postulates that targeting specificity is determined by the interaction between particular members of v- and t-SNARE families. In the nervous system, VAMP-1 acts as a v-SNARE, which binds to two t-SNARE proteins: syntaxin 1 and SNAP-25. Several isoforms of VAMP and syntaxin have been identified outside the nervous system; however, the distribution of two alternatively-spliced variants of SNAP-25, SNAP-25a and SNAP-25b, is restricted to neural and neuroendocrine cells. If the SNARE hypothesis were to be a general model for vesicle docking and membrane fusion, functional equivalents of SNAP-25 should be found in non-neuroendocrine cells. This has prompted a search for a SNAP-25 like protein as well as to examine the tissues distribution and subcellular localization of this novel protein in non-neural cells.
Materials

IMAGE Consortium expressed-sequence tag (EST) cDNA #384468 (Genbank™ accession number H82169) was obtained from Research Genetics Inc. Human melanoma cDNA library, mouse melanocyte melan-b and human melanoma MeWo cell lines were generously provided by Dr. N. Lassam (University of Toronto, Toronto, ON). SNAP-25 monoclonal antibody SMI81 (here called αSN25mAb) was obtained from Sternberger Monoclonals Inc. Anti α1 Na+/K+ ATPase monoclonal antibody 6H was kindly provided by Dr. M. Caplan (Yale University, New Haven, CT). GLUT4 polyclonal antibody was from East Acres Biologicals. An affinity purified antiserum (αSN25pAb) was raised against residues 33-206 of SNAP-25 was a kind gift from Dr. M. Wilson (University of New Mexico, New Mexico) (Schiavo et al., 1993). D-MEM, calf and fetal bovine sera, and other tissue culture reagents were purchased from GIBCO/BRL. Enhanced chemiluminescence detection kit was obtained from Amersham.

Methods

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from tissues and cultured cells using the single-step method of isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Northern blot analysis was performed according to the protocol described in (Sambrook et al., 1989). Total RNA was separated by electrophoresis through a denaturing agarose gel containing 1.2% (v/v) formaldehyde and was then transferred to nylon membranes. RNA was fixed to the membranes by baking for 2 h at 80 °C in vacuum. The membranes were prehybridized with denatured, fragmented salmon sperm DNA at a concentration of 200 μg/ml in prehybridization solution (6X
dCTP-labelled cDNA probe at 42 °C in hybridization solution (6X SSPE, 0.5% SDS, 100 μg/ml salmon sperm DNA, 5% dextran sulfate) in the presence (high stringency) or absence (low stringency) of 50% formamide. Blots were washed for 20 min at room temperature in 1X SSC and 0.1% SDS. This was followed by 3 washes of 20 min each at 65 °C in 0.1X SSC and 0.5% SDS. The RNA of interest was detected by autoradiography.

**cDNA Cloning and Sequencing**

The database of expressed sequence tags (ESTs) was searched by comparing its translated contents to the SNAP-25 amino acid sequence. Translated content of one clone (Genbank™ accession number H82169) was found to be 79% similar and 64% identical to the last 78 residues of SNAP-25. The EST clone labeled with [α-32P]dCTP was used to screen approximately 500,000 plaques from an UNI-ZAP XR human melanoma cDNA library. Twelve positive plaques were identified and their cDNA inserts were subcloned into Bluescript SK(-) phagemids (Stratagene) which after infection with helper bacteriophages, M13KO7, yielded antisense DNA strand. Single-stranded DNA was then used for sequencing using the Sequenase version 2.0 kit (United States Biochemical).

Translation of nucleotide sequences was done with the aid of TRANSLATE algorithm and sequence alignments were performed using the GAP algorithm (Group, 1996). Similar residues are determined using the following convention: K and R; D and E; S and T; F and Y; I, L, V, and M. Percent Identity and Percent Similarity are the respective percentages of symbols that actually match and similar. Symbols that are across from gaps were ignored.
Bacterial Expression of SNAP-23 and SNAP-25 Proteins

The amplified fragment of SNAP-23, with BamH1 and EcoR1 restriction sites engineered at the 5' and 3' ends respectively, was subcloned into pGEX-2T vector and used to transform *Escherichia coli* cells. Transformants were selected using ampicillin resistance. Expression of GST fusion protein of SNAP-23 was induced with 1 mM isopropyl-1-thio-β-D-galactoside (IPTG). Fusion protein was purified by binding to glutathione-agarose beads and subsequent elution using excess amount of reduced glutathione (10 mM). GST fusion protein of SNAP-25 was generated using the same method. The cDNA insert of EST clone (H82169) was isolated and subcloned into the pET-32a expression vector to generate recombinant SNAP-23 protein.

Generation of SNAP-23 Antisera

Two rabbit antisera specific to SNAP-23 were raised. Antibody αSN23.C116 was produced against a recombinant protein encoding carboxy-terminal 116 residues of SNAP-23 (amino acids 96-211) that was liberated from thioredoxin by thrombin cleavage. The serum was affinity purified on a column of Affi-gel 15 to which the GST SNAP-23 fusion protein described above was coupled. Antibody αSN23.C12 was raised against a peptide corresponding to carboxy-terminal residues 200-211 of SNAP-23 (H2N-IANARAKKLIDS-COOH) coupled to keyhole limpet hemacyanin (Biotechnology Service Center, Toronto, ON). An affinity column was produced by coupling the same peptide to SulfoLink Gel (Pierce Chemical Company). Antibodies bound to affinity columns were eluted with 20 mM glycine and 0.2 M NaCl, pH 2.5 and the eluates were neutralized with 0.1 M tris, pH 8.5.
PC12 rat adrenal pheochromocytoma cells were grown in D-MEM with 10% fetal bovine serum. Murine melanocyte cell line, melan-b, and human melanoma MeWo cells were grown in D-MEM supplemented with 2% and 10% fetal bovine serum respectively. A rat muscle cell line, L6, was grown in α-MEM containing 2% fetal bovine serum. Myoblasts were allowed to fuse and differentiate into myotubes upon fusion (Mitsumoto and Klip, 1992). Mouse 3T3-L1 fibroblastic pre-adipocytes (herein referred to as 3T3-L1 fibroblasts) were grown in D-MEM supplemented with 10% calf serum. Cells were allowed to reach confluence, and two days after confluence, monolayers were incubated for 48 h with differentiation medium (D-MEM containing 10% fetal bovine serum, 115 mg/ml isobutylmethylxanthine, 390 ng/ml dexamethasone, and 10 mg/ml insulin) (Student et al., 1980). Monolayers were incubated with D-MEM containing 10% fetal bovine serum and 10 mg/ml insulin for another 48 h. Cells were maintained in D-MEM with 10% fetal bovine serum. All media were supplemented with 100 U/ml of penicillin G sodium and 10 mg/ml streptomycin sulfate. For culturing human melanoma cells, 250 ng/ml amphotericin B was also included.

Membrane isolation

Total membranes (TM) were prepared from confluent cells. All procedures were carried out at 4 ºC. Monolayers were washed with homogenization buffer (20 mM Hepes, 255 mM sucrose, and 1 mM EDTA, pH 7.4), scraped with a rubber policeman in homogenization buffer containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin, and 10 mM E-64), and homogenized with 10 strokes using a cell cracker. Homogenates were centrifuged at 1,000 xg for 5 min. The resultant postnuclear supernatants were centrifuged at 245,000 xg for 90 min to yield total membranes. The supernatants represented the cytosolic fraction.
Tissues and organs were dissected from mice and membranes were prepared according to the protocol described (Nagamatsu et al., 1992). All steps were carried out at 4 °C. Tissues were homogenized in a buffer containing 10 mM Tris/HCl (pH 7.4), 1 mM EGTA, 0.25 mM sucrose, 1 mM phenylmethylsulfonyl fluoride and 1 mM leupeptin, using a Potter homogenizer (20 strokes, 2,000 rpm). The homogenate was centrifuged at 900 xg for 10 min to remove insoluble materials. The supernatant was further centrifuged at 110,000 xg for 75 min. The pellet contained total membranes.

Subcellular Fractionation of 3T3-L1 Adipocytes

Confluent 3T3-L1 adipocytes (7-10 days post-differentiation) were sub-fractionated as described previously (Piper et al., 1991). All procedures were carried out at 4 °C. Monolayers were rinsed and scraped in homogenization buffer containing protease inhibitors. Cells were homogenized by passing them through a cell cracking device as described above. The homogenate was centrifuged at 19,000 xg for 20 min to yield a pellet P1 and supernatant S1. The pellet P1 was resuspended in homogenization buffer, layered on top of a 1.12 M sucrose cushion and centrifuged at 100,000 xg for 1 h. The material at the interface was collected and pelleted by centrifugation at 40,000 xg for 20 min to yield the plasma membrane (PM). The supernatant S1 was centrifuged at 41,000 xg for 20 min and the resulting supernatant S2 was subjected to further centrifugation at 195,000 xg for 75 min to pellet the light density microsomes (LDM). The supernatant was collected as the cytosolic fraction (CYT).

Immunoblotting

Protein samples were solubilized in Laemmli sample buffer (Laemmli, 1970) and separated by SDS-PAGE (Sambrook et al., 1989). Proteins were electrophoretically transferred from gels to polyvinylidene difluoride membranes. These membranes were
Proteins were detected with the following primary antibodies: anti α1 Na+/K+ ATPase monoclonal (1:250); anti SNAP-25 monoclonal αSN25mAb (SMI 81, 1:2,000); anti GLUT4 polyclonal (1:1,000); anti SNAP-25 polyclonal (αSN25pAb, 1:200) which was raised to residues 33-206 of SNAP-25b; affinity-purified SNAP-23 polyclonal antisera αSN23.C116 and αSN23.C12 were used at 4 ng/ml and 0.12 μg/ml, respectively. αSN25pAb was preincubated with bacterial GST protein followed by incubation with glutathione linked to agarose beads to remove any antibodies that may cross-react with the GST portion of SNAP-23 or SNAP-25 fusion proteins before immunoblotting. Enhanced chemiluminescence was used as the method of detection.
Isolation of SNAP-23 from Human Melanoma cDNA Library

In preliminary experiments using a full-length SNAP-25 cDNA probe, I was unable to detect signals corresponding to SNAP-25 in total RNA from 3T3-L1 fibroblasts or adipocytes using high and low stringency hybridization conditions as shown in the left and right panels of Fig. 1 respectively (Wong et al., 1996). In addition, immunoblotting of proteins from 3T3-L1 adipocytes with the monoclonal antibody specific to SNAP-25 (αSN25mAb) failed to give a positive result. However, when plasma membranes prepared from 3T3-L1 adipocytes were probed with a polyclonal SNAP-25 antiserum (αSN25pAb) raised to near full-length SNAP-25 (Schiavo et al., 1993), a polypeptide that migrated at 29 kDa was detected (Fig. 2, left panel). The detection was specific since it was prevented upon preincubation of the antiserum with recombinant SNAP-25 protein (Fig. 2, right panel). These results imply that an immunoreactive species related to SNAP-25 existed in 3T3-L1 cells and prompted us to search for such a gene product.

Through a search of the Genbank™ database, Dr. Trimble identified a human EST clone which encoded a protein predicted to share 64% sequence identity with the C-terminal 78 amino acids of SNAP-25 (Wong et al., 1997). The cDNA (approximately 1.3 Kb) was first used as a probe to screen for expression in different cell lines and tissues at the RNA level. Of the many RNA samples tested, an mRNA species of approximately 2.5 Kb from human melanoma reacted strongly with the probe. This is not surprising since the cDNA insert of the EST clone was originally isolated from human melanocyte, and all the other RNA samples screened were isolated from rodent tissues. The strong detection at RNA level suggested a high probability of cloning a gene product from human melanoma cells, which formed the basis of screening a human melanoma cDNA library. Approximately 500,000 plaques were screened using the same cDNA probe and twelve positive plaques
were isolated. At the time when the twelve clones were being sequenced, a paper appeared which described the identification of a SNAP-25 related gene product, SNAP-23, in human neutrophils (Ravichandran et al., 1996). Sequences of the 5' end of the twelve clones revealed four that were homologous to regions of the SNAP-23 sequence. The longest one (approximately 2.0 Kb) began at nucleotide 50, 43 base pairs upstream from the putative initiation codon of SNAP-23. Comparison between the size of the melanoma clone (2.0 Kb) and the coding region of SNAP-23 (633 bp) indicated that our clone contained the entire coding region of SNAP-23. To amplify the coding region of our clone, PCR technique was employed. Sequence analysis of the PCR product revealed that it was almost identical to SNAP-23 with the exception of two nucleotides: nt 396 (A to G) and nt 404 (T to C) (Fig. 3, bolded letters). The latter nucleotide change results in a different amino acid, Val_{135}→Ala (Fig. 3, bolded letter, and see "Discussion"). The two nucleotide substitutions were not likely to result from errors of PCR (see "Discussion").

The human melanoma SNAP-23 polypeptide contains 211 amino acids with a calculated molecular mass of 23.382 kDa and a theoretical pI of 4.89. Using the GAP algorithm (Genetic Computer Group, 1996) for optimal alignment between amino acid sequences of SNAP-23 and SNAP-25, the two polypeptides were found to be 63% identical and 74% similar to each other (Fig. 4). SNAP-23 exhibits structural characteristics that are similar to SNAP-25. Using the program COILS version 2.1 (Lupas et al., 1991; Lupas, 1996), amino acids 45-79 are predicted to form coiled-coil with probability greater than 80% when scanning the SNAP-23 sequence with both a weighted and unweighted MTIDK matrix. This stretch of amino acids corresponds to one of the three regions in SNAP-25 which have been predicted to form coiled-coil structures. The segments of SNAP-23 that correspond to the other two coiled-coil forming regions of SNAP-25, however, are not likely to form coiled-coils as predicted by the COILS algorithm. Another structural feature of SNAP-23 that resembles SNAP-25 is the cluster
whereas SNAP-25 has four, all of which locate in the middle portion of the two polypeptides. At least one of the four cysteine residues in SNAP-25 has been shown to be palmitoylated, which is believed to be the mechanism responsible for the tight association of SNAP-25 protein with membrane (Oyler et al., 1989; Hess et al., 1992). The presence of a similar cluster of cysteine residues in SNAP-23 may therefore serve the same function.

**Generation of Two SNAP-23 Specific Antibodies**

Two antisera to the C-terminal portion of SNAP-23 were generated. Antibody αSN23.C116 was raised against a truncated portion of the protein (amino acids 96-211) and antibody αSN23.C12 was directed against a peptide corresponding to residues 200-211 of SNAP-23. Equal amounts (1 μg) of GST SNAP-23 and GST SNAP-25 fusion proteins were immunoblotted to test the specificity of the two affinity-purified antisera. Both of these antisera detected only SNAP-23 fusion protein but not SNAP-25 (Fig. 5, top two panels). Moreover, no cross-reactivity to the GST portion of the recombinant protein was detected and no immunoreactivity was detected with the preimmune sera (data not shown). Further characterization of the two antibodies used in preliminary studies is shown in the bottom two panels of Fig. 5. The polyclonal SNAP-25 antiserum, αSN25pAb, cross-reacted to both SNAP-23 and SNAP-25, whereas the monoclonal SNAP-25 antibody, αSN25mAb, was specific for SNAP-25.

**Expression of SNAP-23 protein in different cell lines and tissues**

Ravichandran et al. demonstrated that SNAP-23 is expressed in a variety of non-neuronal tissues at the RNA level (Ravichandran et al., 1996). However, the endogenous expression of the protein was not studied. Therefore I examined the expression of SNAP-23 protein in various cell lines and tissues. SNAP-23 protein was detected using
αSN23.C12 in non-neural cell lines such as mouse 3T3-L1 fibroblasts, 3T3-L1 adipocytes, melan-b melanocytes, rat L6 myotubes, and human melanoma MeWo. Low levels were detected in PC12 neuroendocrine cells (Fig. 6A, top panel). The protein detected by the αSN23.C12 antiserum migrated at approximately 29 kDa. The immunodetection of SNAP-23 was specific since it was blocked by preincubating the antiserum with excess amount of the peptide (Fig. 6A, middle panel). In contrast to the widespread distribution of SNAP-23, SNAP-25 showed a very restricted expression, limited only to the neuroendocrine PC12 cells (Fig. 6A, bottom panel).

As shown above for cell lines, SNAP-23 was also widely distributed in different tissues from rats and mice (Fig. 6B). The protein was found to be abundant in lung, liver, spleen and kidney, but relatively less concentrated in rat skeletal muscle and mouse heart. Upon longer exposure, a low level of SNAP-23 could also be detected in the mouse brain (not shown).

**Subcellular localization of SNAP-23 in 3T3-L1 cells**

The subcellular distribution of SNAP-23 is not known, but like SNAP-25, it lacks a stretch of hydrophobic residues necessary to form a transmembrane domain. In order to determine the localization of SNAP-23 in 3T3-L1 fibroblasts we used subcellular fractionation. The results in Fig. 7A show that SNAP-23 was exclusively found in the membrane fraction (TM) with no detectable amount found in the cytosolic fraction (CYT). Since SNAP-23 also contains a cluster of cysteine residues, it may associate with the membrane by palmitoylation of one or more of the cysteines. 3T3-L1 fibroblasts can be differentiated into adipocytes, hence allowing me to study the protein at two stages of differentiation. Proteins that are involved in intracellular traffic have been shown to be expressed at different levels following differentiation into adipocytes. For example, cellubrevin, a member of the synaptobrevin family, is expressed predominantly in
abundant at the fibroblast stage (Volchuk and Klip, unpublished observation). As can be seen in Fig. 7B, there was no significant change in the level of expression of SNAP-23 upon differentiation, suggesting that the protein is necessary in both fibroblasts and adipocytes.

The presence of SNAP-23 in 3T3-L1 adipocytes provided us with an opportunity to further study the subcellular distribution of the protein. This is because the fractionation protocol for 3T3-L1 adipocytes has been well-established (Piper et al., 1991). By this procedure, the plasma membrane (PM), light density microsomes (LDM), and cytosol (CYT) were readily purified. The α1 subunit of Na⁺/K⁺ ATPase is a useful marker for the PM. This protein was detected almost exclusively in the PM and not in the LDM or CYT of 3T3-L1 adipocytes (top panel of Fig. 7C). The LDM contained the majority of the GLUT4 glucose transporter which locates intracellularly in unstimulated cells (Fig. 7C, middle panel). The majority of SNAP-23 was found to be associated with the PM (Fig. 7C, bottom panel). Occasionally, a small amount of SNAP-23 was also detected in the LDM (data not shown).

**Subcellular distribution of SNAP-23 in non-neural secretory cells**

The presence of SNAP-23 and its subcellular distribution was also examined in non-neural secretory cells such as white blood cells, specifically human neutrophils and the pancreatic acinar cells. These studies were done in collaboration with Dr. S. Grinstein and Dr. H. Y. Gaisano respectively. To search for the presence of SNAP-23 in different types of white blood cells, platelets, lymphocytes, monocytes and neutrophils were isolated from heparinized blood from healthy individuals by John Brumell in Dr. Grinstein's laboratory. Lysates were prepared from these cells and proteins, and I used them for immunoblot analysis using the SNAP-23 antibody, αSN23.C12 (Fig. 8). Consistent with the
Observations made above, SNAP-23 was not detected in brain microsomes but was expressed in 3T3-L1 adipocytes (Fig. 8, lanes 1 and 2). The protein was also detected in abundance in lymphocytes, monocytes and neutrophils (Fig. 8). This observation had prompted us to look further at the subcellular distribution of SNAP-23 in neutrophils. The three types of granules (primary, secondary and tertiary), the secretory vesicles and plasma membrane fraction, as well as the cytosol were obtained from Dr. N. Borregaard (Rigshospitalet, Denmark) following fractionation of human neutrophils (Kjeldsen et al., 1993). Equal amount of protein (25 μg) from each fraction was subjected to immunoblot analysis and a representative blot was shown in Fig. 9. SNAP-23 was predominantly associated with the secretory vesicle/plasma membrane fraction with barely detectable levels in secondary and tertiary granules (Fig. 9). The positive detection of SNAP-23 in granules may result from contamination of the granular fractions with secretory vesicles and plasma membrane. Preliminary results using separated secretory vesicles and plasma membranes indicate that SNAP-23 is associated primarily with the plasma membranes but not secretory vesicles (data not shown).

The second non-neural secretory cell type which was tested for the presence of SNAP-23 was pancreatic acinar cells. In order to determine the presence and subcellular distribution of SNAP-23 and/or SNAP-25 in pancreatic acinar cells, purified plasma membrane (PM) and zymogen granule membrane (ZGM) were isolated from rat pancreas in Dr. Gaisano's laboratory. To avoid contamination of SNAP-25, which is abundant in endocrine pancreas, with the exocrine pancreatic acini, streptozotozin-treated rats were used. This treatment specifically destroyed the β-cells of the endocrine pancreas. SNAP-23 was detected with SNAP-23 peptide antibody αSN23.C12, and as expected, in membranes from 3T3-L1 fibroblasts (3T3) but not in brain microsomes (Fig. 10, first row). Moreover, SNAP-23 was also detected in PM and ZGM of pancreatic acinar cells. The immunodetection was specific for SNAP-23 since preincubation of the antibody with
excess amount of GST-SNAP-23 fusion protein had no effect on the detection (Fig. 10, second row); however, the bands disappeared when the antibody was preincubated with GST-SNAP-23 fusion protein (Fig. 10, third row). The presence of SNAP-25 was also examined in the purified fractions of pancreatic acinar cells. An affinity-purified antibody that was raised against the full-length fusion protein of SNAP-25 was used for the immunodetection. The antibody reacted strongly with SNAP-25 present in rat brain and gave a weaker signal in PM and ZGM from pancreatic acini, and membranes from 3T3-L1 fibroblasts (Fig. 10, fourth row). Preincubation of SNAP-25 antibody with GST-SNAP-23 fusion protein had no effect on the band in rat brain, but abolished the signal in PM, ZGM, and 3T3 (Fig. 10, fifth row). All the signal disappeared when the antibody was preincubated with GST-SNAP-25 fusion protein (Fig. 10, bottom row). These results suggest that an immunoreactive species that was similar but not identical to SNAP-25 was present in pancreatic acinar cells. Furthermore, the SNAP-25 antibody cross-reacted with both SNAP-23 and SNAP-25 proteins. Taking together all the observations, SNAP-23 was present in both the plasma membrane and the zymogen granule membrane of pancreatic acinar cells.
Fig. 1. A SNAP-25 cDNA probe did not hybridize with RNA from 3T3-L1 cells. Twenty micrograms of total RNA from brain, 3T3-L1 adipocytes (3T3-L1 A), and fibroblasts (3T3-L1 F) was analyzed by Northern blot analysis. Hybridization was carried out under both high (left panel) and low (right panel) stringency conditions with a full-length SNAP-25 cDNA probe. 28S and 18S referred to the locations of the ribosomal RNAs. An arrow indicated SNAP-25 mRNA and its expected size is 2.2 Kb.
Fig. 2. Immunodetection of a SNAP-25 like protein in membranes from 3T3-L1 adipocytes. Two micrograms of brain microsomes, equal amounts (30 µg) of plasma membranes (3T3-L1 A PM) and light density microsomes (3T3-L1 A LDM) from 3T3-L1 adipocytes were subjected to SDS-PAGE and immunblotted with either an affinity purified antiserum raised to residues 33-206 of SNAP-25, αSN25pAb (left panel) or antiserum that was preincubated with excess amount of GST fusion protein of SNAP-25 (right panel).
**Fig. 3.** Nucleotide sequence of human melanoma SNAP-23 and its predicted amino acid sequence. The amino acid sequence of human melanoma SNAP-23 is represented in single-letter code deduced from the nucleotide sequence shown above. The bold letters represent an amino acid and nucleotides that are different from the recently identified SNAP-23 in human neutrophils (Ravichandran et al., 1996). The two nucleotide changes are at nt 396 (A to G) and nt 404 (T to C). The latter difference results in a change in amino acid, Val^{135}→Ala.
Fig. 4. Comparison of amino acid sequences of human melanoma SNAP-23 and SNAP-25B. The two sequences were aligned using the GAP algorithm (Group, 1996). A pipe character (|) indicates identical amino acids. Similar residues are indicated by a colon (: ) using the following convention: K and R; D and E; S and T; F and Y; I, L, V, and M. The two sequences are 63% identical and 74% similar.
Fig. 5. Characterization of SNAP-23 and SNAP-25 antibodies. Immunoblot analysis was used to test the specificity of four antibodies to either SNAP-23 or SNAP-25 GST fusion proteins. One microgram of each fusion protein was subjected to SDS-PAGE and immunoblotted with the following antibodies: αSN23.C116 affinity purified antiserum raised to the C-terminal 116 amino acids of SNAP-23; αSN23.C12 affinity purified antiserum raised to the C-terminal 12 amino acids of SNAP-23; αSN25pAb polyclonal antiserum raised to residues 33-206 of SNAP-25b; and αSN25mAb (SM1 81) monoclonal antibody to SNAP-25.
Fig. 6. Expression of SNAP-23 in cell lines and tissues. (A) Immunoblot of total membranes prepared from various cell lines of different species. Twenty micrograms of proteins from rat neuroendocrine PC12 cells, rat L6 myotubes (L6 Mt), mouse 3T3-L1 fibroblasts (3T3-L1 F) and 3T3-L1 adipocytes (3T3-L1 A), mouse melanocytes (melan-b), and human MeWo melanoma cell line were resolved by SDS-PAGE and probed for SNAP-23 with αSN23.C12 antibody (0.12 μg/ml) (top). The specificity of immunodetection was confirmed by preincubation of αSN23.C12 antiserum with excess amount peptide (0.2 μg/ml) (middle). Detection of SNAP-25 by the monoclonal antibody αSN25mAb (SM1 81) was restricted to neuroendocrine PC12 cells (bottom). (B) Immunoblotting of total membranes from rat skeletal muscle (Sk. muscle) and other tissues from mouse with αSN23.C12 antibody.
Fig. 7. Localization of SNAP-23 in 3T3-L1 cells. (A) Twenty micrograms of total membranes (TM) and cytosolic fraction CYT) from 3T3-L1 fibroblasts (3T3-L1 F) were subjected to immunoblotting using the αSN23.C12 antibody. (B) Twenty micrograms of total membranes (TM) from 3T3-L1 fibroblasts (3T3-L1 F) and adipocytes (3T3-L1 A) were immunoblotted for SNAP-23 using αSN23.C12 antibody. (C) To study the subcellular distribution of SNAP-23 in 3T3-L1 adipocytes (3T3-L1 A), cells were subfractionated and equal amounts (15 μg) of plasma membrane (PM), light density microsomes (LDM), and cytosolic fraction (CYT) were separated by SDS-PAGE and probed for α1 subunit of Na⁺/K⁺ ATPase with 6H monoclonal antibody, GLUT4 with a polyclonal antiserum, and SNAP-23 with αSN23.C12 antibody.
Fig. 8. Expression of SNAP-23 in different types of white blood cells. Isolation of different white blood cell types from fresh heparinized blood of human was performed by John Brumell in Dr. S. Grinstein's laboratory. Thirty micrograms of protein from mouse brain microsomes and 3T3-L1 adipocyte total membranes were used as negative and positive control for SNAP-23. Lysates prepared from $10^6$ cells of platelet, lymphocyte, monocyte and neutrophil were subjected to immunoblot detection for the presence of SNAP-23 using $\alpha$SN23.C12 antibody.
Fig. 9. Subcellular distribution of SNAP-23 in human neutrophils. Fractionation of human neutrophils were done in Dr. N. Borregaard's laboratory and the following fractions were obtained: primary, secondary and tertiary granules; secretory vesicles and plasma membrane fraction (sv/pm); and cytosol. Twenty-five micrograms of protein from each of the fractions were subjected to 12% SDS-PAGE. The presence of SNAP-23 were detected using the SNAP-23 antibody, αSN23.C12.
Fig. 10. Distribution of SNAP-23 in pancreatic acinar cells. Highly purified pancreatic acinar plasma membranes (PM, 10 μg of protein) and zymogen granule membrane (ZYM, 10 μg of protein) from diabetic (streptozotocin-treated) rats, crude brain homogenates (rat brain, 5 μg of protein) and 3T3-L1 fibroblast microsomes (3T3, 10 μg of protein) were prepared, electrophoresed on a 15% SDS-polyacrylamide gel and blotted on Immobion-P transfer membranes. These were then immunoblotted with the following antibodies: top row - αSN23.C12 SNAP-23 antibody; second row - αSN23.C12 antibody preincubated with 1 μg of GST SNAP-23 fusion protein; third row - αSN23.C12 antibody preincubated with 1 μg of GST SNAP-23 fusion protein; fourth row - SNAP-25 antibody (Anti-SNAP-25) that was raised against the full-length fusion protein of SNAP-25; fifth row - Anti-SNAP-25 preincubated with 1 μg of GST-SNAP-23; and bottom row - Anti-SNAP-25 preincubated with 1 μg of GST-SNAP-25.
**SNAP-23 is a SNAP-25-related gene product**

I have described here the identification of a novel protein composed of 211 amino acids, which is 63% identical and 74% similar to SNAP-25. At the time when I was sequencing all the potential clones resulting from screening a human melanoma cDNA library, a paper was published describing the identification of a SNAP-25-related protein, SNAP-23. One of our clones contained the entire coding region of SNAP-23, and alignment of the coding region of the two SNAP-23 sequences revealed only two nucleotide substitutions which are highlighted in Fig. 3. The change at nt 396 gives rise to synonymous codons that specify the same amino acid. However the change at nt 404 results in a different amino acid, from Val to Ala. Both Val and Ala are hydrophobic amino acids, and so is the corresponding amino acid Ile in SNAP-25 (Fig. 4), therefore these amino acid changes are not likely to drastically alter the secondary structure of these proteins. The two nucleotide changes that were observed could not be attributed to errors in our sequencing since the exact changes are documented in a recent paper which describes the identification of two isoforms of SNAP-23 in human neutrophils (Mollinedo and Lazo, 1997). One of the isoforms, SNAP-23A, contains the exact nucleotide substitutions as observed in the melanoma clone. The other isoform, SNAP-23B, has a deletion of 159 nucleotides in the middle portion of SNAP-23 from nt 266 to nt 424, and is predicted to encode a protein containing 158 residues (discussed later). The nucleotide differences observed in both SNAP-23A and the melanoma SNAP-23 clone can be attributed to either polymorphism among humans caused by spontaneous mutations that arise randomly in the nucleotide sequences of genes or to sequencing errors made by Ravichandran et al. in the original SNAP-23 sequence (Ravichandran et al., 1996).
Other than its high level of sequence similarity to SNAP-25, SNAP-23 also exhibits several features that are characteristic of SNAP-25. According to the SNARE hypothesis, SNAP-25 and syntaxin on the target membrane (t-SNARE) form a complex with VAMP/synaptobrevin on the vesicle membrane which is crucial for mediating vesicle fusion with the target membrane. Binary interactions among the three SNAREs have been characterized in vitro. Furthermore, a detergent-insoluble ternary complex of these three proteins has been isolated from presynaptic nerve terminals (Sollner et al., 1993b). Ravichandran et al. characterized the affinity of the in vitro translated SNAP-23 protein for syntaxin isoforms 1-4 as well as VAMPs 1 and 2, and found them to be similar to that observed for SNAP-25 (Ravichandran et al., 1996). The interactions among the SNARE proteins are believed to be mediated through coiled-coils. SNAP-25 has three regions with a high probability of coiled-coil formation. Using the COILS version 2.1, the three regions that are predicted to form coiled-coils with probability higher than 80% are residues 1-39, 49-80, and 166-202 (Lupas et al., 1991; Lupas, 1996). SNAP-23 has only one region with high probability of forming coiled-coil and it spans residues 45-79 (> 80% probability) as determined by the COILS algorithm (Lupas et al., 1991; Lupas, 1996). Since the coiled-coil domains in SNAP-25 are not completely conserved in SNAP-23, the association of SNAP-23 with syntaxin and VAMP isoforms is predicted to be different from that of SNAP-25.

A second structural feature of SNAP-25 that is conserved in SNAP-23 is the cluster of cysteine residues located in the middle portion of the protein spanning residues 79-87 (Fig. 3). SNAP-25 protein is an intrinsically hydrophilic polypeptide since its amino acid sequence lacks a stretch of hydrophobic residues that are characteristic of transmembrane regions. However, fractionation of hippocampal extracts has revealed that SNAP-25 is tightly-associated with the synaptosomal membrane fraction and it cannot be extracted by concentrated salt washes unless detergent is added (Oyler et al., 1989). The association of
SNAP-25 protein with membranes is probably via palmitoylation of one or more of the four cysteine residues which span amino acids 84-92 (Hess et al., 1992). It has been shown that deletion of a stretch of 12 amino acids encompassing the four cysteine residues renders the deletion mutant of SNAP-25 completely soluble and resistant to [3H] palmitic acid labeling (Veit et al., 1996). Since SNAP-23 also contains a similar cluster of cysteine residues, it may associate with the membrane by a similar mechanism. Interestingly, SNAP-23 contains five cysteine residues that are in the same arrangement as the *Torpedo* SNAP-25 sequence (Risinger et al., 1993a). In contrast, the mammalian SNAP-25a and SNAP-25b have each a substitution of one of the cysteines, breaking the cluster arrangement. The extra cysteine residue found in SNAP-23 and *Torpedo* SNAP-25 may define an ability to associate with specific proteins and/or affect its ability and efficiency of acylation.

The functional importance of SNAP-25 is highlighted by the discovery that the protein is a proteolytic substrate of *Clostridium botulinum* neurotoxin types A, E (Schiavo et al., 1993; Blasi et al., 1993a; Binz et al., 1994; Lawrence et al., 1994; Boyd et al., 1995; Pellegrini et al., 1995; Schmidt and Bostian, 1995), and C1 (Foran et al., 1996). Cleavage of SNAP-25 by BoNT/A or BoNT/E results in an inhibition of neurotransmitter release from isolated nerve terminals (Blasi et al., 1993a), a blockade of stimulated insulin release from pancreatic β cells (Boyd et al., 1995), and an abrogation in catecholamine secretion by adrenal chromaffin cells (Lawrence et al., 1994). Sequence alignment of SNAP-23 and SNAP-25 proteins reveals that the two amino acids which constitute the proteolytic cleavage site for BoNT/E but not those for BoNT/A are conserved in SNAP-23 (Fig. 4). Therefore we predict that SNAP-23 may be protected from proteolytic cleavage by BoNT/A. BoNT/E may be a useful tool to discern whether SNAP-23 plays a role in exocytosis. Indeed, we have shown that both SNAP-23 and SNAP-25 are present in PC12 cells; in these cells, exocytosis of large dense core vesicles is inhibited completely by
BoNT/E but not BoNT/A, although both toxins effectively cleaved SNAP-25 (Banerjee et al., 1996). The presence of SNAP-23 and its potential insensitivity to proteolysis by BoNT/A may explain the incomplete inhibition in the peptide hormone secretion observed in PC12 cells. However, it remains to be proven that BoNT/E acts on SNAP-23, and indeed the residues flanking the cleavage site in SNAP-25 are not reproduced in SNAP-23.

Expression of SNAP-23 in different tissues and cells

Preliminary experiments using a polyclonal antibody, αSN25pAb, which was raised against a recombinant protein encompassing residues 33-206 of SNAP-25, detected a protein that migrated at 29 kDa in the plasma membrane fraction of 3T3-L1 adipocytes. The detection was specific since it was prevented upon preincubation of the antiserum with recombinant SNAP-25 protein (Fig. 2). However, immunoblotting of proteins from 3T3-L1 adipocytes with a monoclonal antibody specific to SNAP-25 (αSN25mAb) failed to detect the signal. Furthermore, using a full-length SNAP-25 cDNA as probe, RNA blot analysis was unable to detect a signal corresponding to SNAP-25 in 3T3-L1 adipocytes under both high and low stringency hybridization conditions (Fig. 1). Taking together all these observations, we concluded that an immunoreactive species related to SNAP-25 exists in 3T3-L1 cells. These results also imply that the polyclonal SNAP-25 antiserum, αSN25pAb, cross-reacted to both neural-specific SNAP-25 and the related protein expressed in 3T3-L1 adipocytes. Indeed, αSN25pAb reacts with both SNAP-23 and SNAP-25 fusion proteins (Fig. 5, third panel), whereas the monoclonal αSN25mAb is specific for SNAP-25 (Fig. 5, bottom panel). These data indicate that the immunoreactive protein detected by αSN25pAb in 3T3-L1 adipocytes was most likely SNAP-23 but not SNAP-25.

In contrast to our inability to detect SNAP-25 in 3T3-L1 adipocytes, L6 muscle cells and rat skeletal muscle (Volchuk et al., 1994), Jagadish et al. report the detection of
SNAP-25a cDNA in fat tissues and SNAP-25b cDNA in skeletal muscle by polymerase chain reaction which amplifies DNA template using isoform-specific primers (Jagadish et al., 1996). Polymerase chain reaction is a very sensitive technique in detecting low level of DNA template, however, presence of trace amounts of DNAs that can serve as template can also contaminate the reaction mixture and give false positive detection. The detection of SNAP-25 cDNA in fat and muscle tissues could potentially result from the presence of nerve terminals, which are enriched in SNAP-25, in the biological material. Alternatively, the amount of SNAP-25 protein in our fat and muscle cell lines may be below the detection level of our antibodies.

Unlike SNAP-25 whose distribution is restricted to neural and neuroendocrine cells, SNAP-23 protein was found to be expressed by all non-neuronal cell types and tissues tested (Fig. 6). Interestingly, the immunoreactive bands from cultured L6 myotubes (L6 Mt, Fig. 6A) and heart (Fig. 6B) appear as a doublet. There are three potential explanations for this observation: first, the doublet can represent SNAP-23 protein in two phosphorylation states; second, the doublet can indicate the presence of two isoforms of SNAP-23 of similar size in muscle cells; third, the antibody may cross-react with a SNAP-23 related gene product of similar size which remains to be identified.

**Subcellular localization of SNAP-23 in non-neural cells**

To investigate the subcellular distribution of endogenous SNAP-23 protein, total membrane and cytosolic fractions were obtained from undifferentiated cultured fibroblastic cells. Fig. 7A shows a representative immunoblot where SNAP-23 associated entirely with membranes (3T3-L1 F TM) and was undetectable in the soluble fraction (CYT). The distribution of SNAP-23 is reminiscent of SNAP-25 in which the majority of the protein is bound tightly to the synaptosomal membrane (Oyler et al., 1989). As stated above, it is
plausible that SNAP-23 associates with the membrane by a similar acylation mechanism, although this needs to be confirmed experimentally.

The finding that SNAP-23 protein was expressed in differentiated 3T3-L1 adipocytes (Fig. 7B) allowed further characterization of the subcellular localization of the protein in these cells. Fig. 7C demonstrates clearly the preferential localization of SNAP-23 at the plasma membrane. This is consistent with the idea of it being a t-SNARE protein along with syntaxin 4 which has also been detected at the cell surface of 3T3-L1 adipocytes (Volchuk et al., 1996). Preliminary immunofluorescence studies carried out by Dr. P. J. Bilan using our affinity-purified SNAP-23 peptide antibody αSN23.C12, showed a signal not only at the perimeter of 3T3-L1 adipocytes but also intracellular staining. This appears to contradict the exclusive distribution of SNAP-23 in the plasma membrane fraction of 3T3-L1 adipocytes indicated by the immunoblot analysis as shown in Fig. 7C. The apparent inconsistency between the two methods of detection might be explained by the existence SNAP-23B. The SNAP-23B cDNA contains a deletion of 159 nucleotides which results in a truncated SNAP-23 protein: amino acids 89-142 are replaced by a Ser residue which joins the N- and C-terminal fragments of SNAP-23 (Mollinedo and Lazo, 1997). SNAP-23B contains 158 residues and the predicted molecular mass is 17.8 kDa. The level of expression of SNAP-23B protein in cells remains to be determined. An immunoblot of subcellular fractions of 3T3-L1 adipocytes using αSN23.C12, which should detect both the full-length SNAP-23 (SNAP-23A) and SNAP-23B, showed a positive detection of a protein which migrated at about 19 kDa in the cytosolic fraction but not associated with membranes (data not shown). Although this observation needs to be confirmed by future experiments, the presence of an isoform of SNAP-23 which is mainly soluble could explain the intracellular staining of SNAP-23 observed in the immunofluorescence images of 3T3-L1 adipocytes. Alternatively, the intracellular staining could be attributed to non-specific binding of the antibody to cellular components.
To investigate whether the SNARE hypothesis can indeed serve as a general model for intracellular vesicle traffic, the expression of SNAP-23, a non-neural equivalent of SNAP-25 (t-SNARE), was investigated in two additional non-neural secretory cell types: white blood cells and pancreatic acinar cells. In contrast to 3T3-L1 adipocytes in which insulin causes incorporation of vesicle membrane-bound glucose transporters to the cell surface, white blood cells and pancreatic acinar cells secret digestive enzymes via exocytosis of the luminal content of granules to the extracellular mellieu in response to stimuli. Four types of white blood cells were screened for the presence of SNAP-23. Lymphocytes, monocytes, neutrophils and platelets, all were found to express SNAP-23 (Fig. 8). The subcellular localization of SNAP-23 in neutrophils was examined further. Polymorphonuclear neutrophils protect the body against invading organisms such as bacteria, by attacking and destroying them. The initial step of the process involves phagocytosis of the infectious agent. Once the foreign particle was phagocytosed, lysosomes and other granules dock and fuse with the phagocytic vesicle (plasma membrane vesicle located intracellularly) and subsequently fuse with it, thereby allowing release of digestive enzymes and bactericidal agents into the phagosome (Guyton, 1992). The SNARE proteins syntaxin 4 and VAMP-2, but not SNAP-25, have been detected in neutrophils (Brumell et al., 1995). Fractionation of neutrophils yields the cytosolic, and three granule fractions (primary, secondary, and tertiary) as well as a membrane fraction containing secretory vesicles and plasma membrane (sv/pm). Immunoblot of these fractions indicated that SNAP-23 is almost exclusively associated with sv/pm (Fig. 9). Upon further separation of secretory vesicles from plasma membrane, results from one experiment indicated that SNAP-23 was detected only in the plasma membrane fraction (data not shown). The localization of SNAP-23 at the plasma membrane is suggestive that the protein acts as a t-SNARE. Previous studies have shown that syntaxin 4 is localized exclusively at the plasma membrane of neutrophils, whereas VAMP-2 is detected on the
secretory vesicle and tertiary granule (Brumell et al., 1995). The identification of SNAP-23 in neutrophils suggest that SNAP-23 and syntaxin 4 are likely to act as t-SNAREs, with VAMP-2 being the v-SNARE. Indeed upon activating neutrophils with Ca\(^{2+}\) ionophore, VAMP-2 is found to redistribute to the plasma membrane (Brumell et al., 1995). Granular secretion of bactericidal agents into the phagosome could be achieved by interaction of SNAP-23 and syntaxin 4 on the phagosomal membrane (plasma membrane) with VAMP-2 on the granule, followed by vesicle fusion with the phagosome.

Unlike adipocytes and white blood cells, pancreatic acinar cells are polarized epithelial cells. The plasma membrane of the acinar cells is organized into two discrete regions: apical and basolateral. The extracellular spaces are segregated by tight junctions which are essential to maintain the functional integrity of the exocrine pancreas. Pancreatic acinar cells undergo regulated secretion of digestive enzymes which are stored in zymogen granules (ZG), upon stimulation with molecules such as acetylcholine, gastrin and cholecystokinin (Guyton, 1992). Previous studies have identified VAMP-2 (Gaisano et al., 1994) and syntaxin isoforms 1-4 in pancreatic acinar cells (Gaisano et al., 1996). In a collaborative study with Dr. Gaisano, the subcellular distribution of SNAP-23 was examined in pancreatic acinar cells. Surprisingly SNAP-23 was detected by immunoblotting in both the plasma membrane (PM) and zymogen granular membrane (ZGM) fractions (Fig. 10, top panel). The detection was specific since it was inhibited upon preincubation of SNAP-23 peptide antibody, αSN23.C12, with SNAP-23 fusion protein (Fig. 10, third panel), and was not affected by preincubation of the antibody with excess amount of SNAP-25 fusion protein. (Fig. 10, second panel). The presence of SNAP-23 in the ZGM was not likely to be caused by contamination of this fraction with plasma membrane since the intensities of both signals were almost identical. The presence of SNAP-23 on the ZGM could mean that the ZG is the target for fusion of other intracellular vesicles. This idea is supported by the observation of ZG-ZG fusion (Scheele
et al., 1987). Of the four isoforms of syntaxin present in the pancreatic acini, syntaxin 3 was present on ZG (Gaisano et al., 1996). Therefore, syntaxin 3 along with SNAP-23 could act as t-SNARE on ZG. VAMP-2 is the only v-SNARE found to be localized on ZG. In vitro binding studies have demonstrated that VAMP-1 and -2 bind only to syntaxin 1 and 4 but not 2 and 3 (Calakos et al., 1994), whereas SNAP-23 can bind to all four isoforms of syntaxin (Ravichandran et al., 1996). Therefore ZG-ZG fusion can be achieved either by binding of VAMP-2 to SNAP-23 or a yet-to-be-identified v-SNARE which can interact with both syntaxin 3 and SNAP-23.

The results presented in this chapter described the identification of SNAP-23 - a novel isoform of SNAP-25. In contrast to SNAP-25 which is expressed only in neural and neuroendocrine cells, SNAP-23 is distributed in a variety of cells and tissues. Subcellular fractionation of 3T3-L1 adipocytes, human neutrophils, and pancreatic acinar cells demonstrated that SNAP-23 is localized predominantly at the plasma membrane which is suggestive of its role in being a t-SNARE along with syntaxin 4 on the plasma membrane.
The SNARE hypothesis was initially proposed to explain how synaptic vesicles dock and fuse with the presynaptic plasma membrane, allowing transmitter release at the nerve terminals. Homologues of proteins responsible for this highly regulated exocytic process at the nerve terminals are found to be necessary for the constitutive exocytosis in yeast. Isoforms of syntaxin and VAMP, which are key components of the protein machinery for vesicle transport, have also been identified in mammalian non-neural tissues and cells. Furthermore, the functions of syntaxin and VAMP have been implicated in regulated exocytosis in both neural and non-neural cells. From these data emerges the theme of universality of the SNARE hypothesis which predicts that all eukaryotic cells have families of v- and t-SNAREs, whose members form cognate pairs that define binding specificity that is essential for vesicle docking and fusion in vivo (Rothman, 1994). SNAP-25 is a crucial component of the protein machinery important for vesicle docking and fusion. Prior to my M.Sc. project, expression of SNAP-25 and its function had only been characterized in the nervous system and neuroendocrine cells. According to the SNARE hypothesis, functional equivalents of SNAP-25 must exist outside the nervous system to allow regulated exocytosis to occur in non-neural tissues.

The aim of this Thesis was to search for a non-neural equivalent of SNAP-25 that can explain regulated exocytosis in cells outside the nervous system. The search led to the identification of a novel polypeptide which shares 63% identity to SNAP-25. This novel protein was found to be identical to SNAP-23 which was published at the same time when the sequences of our cDNA clones resulted from screening a human melanoma cDNA library were being analyzed. SNAP-23 is expressed in a variety of tissues and cell lines including non-neural cells such as adipocytes, muscles, neutrophils and pancreatic acinar cells as well as neuroendocrine PC12 cells. This ubiquitous distribution of SNAP-23 was
expression of SNAP-23 at the mRNA level (Ravichandran et al., 1996). Fractionation studies of murine 3T3-L1 adipocytes and human neutrophils suggest that SNAP-23 is exclusively associated with the plasma membrane. SNAP-23, along with syntaxin 4 which is primarily localized at the plasma membrane of 3T3-L1 adipocytes (Volchuk et al., 1996) and human neutrophils (Brumell et al., 1995), may act as a t-SNARE. The corresponding v-SNARE, both on the GLUT4 vesicles in 3T3-L1 adipocytes (Volchuk et al., 1995) and in secretory vesicles and tertiary granules of human neutrophils (Brumell et al., 1995), is VAMP-2. In rat pancreatic acinar cells, SNAP-23 is found on the plasma membrane as well as the zymogen granular membrane. This observation indicates that some of the SNAP-23 protein acts as a t-SNARE on the plasma membrane. In addition, rat pancreatic acinar cells possess another pool of SNAP-23 that is localized intracellularly, suggesting that zymogen granule may act as the target membrane for vesicle fusion. Now that a new player for vesicle traffic has been identified, future work is needed to establish the functional importance of the protein in regulated exocytosis underlying insulin-stimulated translocation of GLUT4-containing vesicles in 3T3-L1 adipocytes and secretion in human neutrophils and rat pancreatic acinar cells.

One of the important tools in discerning the function of SNAP-25 is one of the most potent neurotoxins known to mankind: Botulinum neurotoxin (BoNT). Both BoNT types A and E cleave SNAP-25 at distinct sites as indicated in Fig. 4. The two residues composing the BoNT/E cleavage site, Arg\textsuperscript{180} - Ile\textsuperscript{181}, are conserved in SNAP-23. However, the BoNT/A cleavage site is not conserved since Gln\textsuperscript{197} in SNAP-25 is replaced by Ala in SNAP-23; therefore, SNAP-23 is unlikely to be a substrate for proteolysis by BoNT/A. It will be important to test the susceptibility of SNAP-23 to cleavage by BoNT/E. Since the cleaved product of SNAP-23 will lack the C-terminal 25 residues which contains the epitope of the SNAP-23 peptide antibody \(\alpha_{SN23.C12}\), immunoblotting
intact SNAP-23 that is resistant to cleavage.

Different experiments can be used to establish a role of SNAP-23 in non-neural cells. In 3T3-L1 adipocytes where insulin stimulates the translocation of intracellular pool of GLUT4 proteins, SNAP-23 antibodies (αSN23.C12) and BoNT/E can either be microinjected into intact cells or incubated with permeabilized adipocytes to interfere with the normal function of SNAP-23 prior to insulin treatment. The inhibition can be measured by either arrival of GLUT4 protein on the cell surface detected with GLUT4 antibody or, in the case of permeabilized adipocytes, insulin-stimulated glucose transport using radioactively-labelled 2-deoxyglucose. Furthermore, C-terminal SNAP-25 peptide fragments of 12-mer and 20-mer in length have been used to inhibit the regulated exocytosis in adrenal chromaffin cells and synaptosomes (Gutierrez et al., 1995; Mehta et al., 1996; Gutierrez et al., 1997). The C-terminus of SNAP-25 has been suggested to be crucial for binding to VAMP-1. If similar interaction occurs between SNAP-23 and VAMP-2, incubation of electroporated neutrophils with excess amount of C-terminal 12-mer of SNAP-23 may interfere with the secretion of granules. The peptide is predicted to bind to most of the native VAMP proteins, thereby preventing the normal v- and t-SNARE interactions necessary for vesicle fusion. In rat pancreatic acinar cells, the function of SNAP-23 can be assessed by the effect that introducing SNAP-23 antibody or BoNT/E into permeabilized cells may have on the Ca²⁺-stimulated enzyme secretion.

As stated earlier, two isoforms of SNAP-23 were recently identified in human neutrophils (Mollinedo and Lazo, 1997). Preliminary results suggested that the SNAP-23 peptide antiserum, which should detect both SNAP-23A and SNAP-23B, reacted with a polypeptide that migrates at 19 kDa in the cytosolic fraction of 3T3-L1 adipocytes and the detergent-solubilized lysate of human neutrophils. If SNAP-23B protein were indeed expressed by these cells, it would be important to study its interaction with syntaxin 4 (t-
interactions would suggest a new regulatory role of SNAP-23B in vesicle traffic in adipocytes and neutrophils. It is easy to envisage SNAP-23B as a fusion clamp in the non-stimulated state, for example, if it has binding capacity for either syntaxin 4 or VAMP-2 but not both. Future study can be designed to investigate the in vitro binding properties of SNAP-23B with the other SNARE proteins.

Recent work by Wang et al. described the identification of a SNAP-25 related protein, syndet, in murine 3T3-L1 adipocytes (Wang et al., 1997). An important question arises immediately - is syndet a mouse isoform of SNAP-23? The similarities and differences between syndet and SNAP-23 are discussed here. Syndet is 58% similar to SNAP-25, a value which is much lower than the 74% similarity shared between SNAP-23 and SNAP-25. The mouse syndet and human SNAP-23 amino acid sequences share only 88% identity, unlike mouse and human isoforms of SNAP-25 which are completely identical. This may suggest the two proteins represent distinct polypeptides rather than being species-specific isoforms of the same protein. Furthermore, the three regions that are highly probable in coiled-coils formation in SNAP-25 (two at the amino-terminus and one at the carboxyl-terminus) are not conserved in either SNAP-23 or syndet. Using the COILS algorithm, only the second coiled-coil is conserved in SNAP-23, whereas for syndet, both the second and third coiled-coil regions are conserved. The coiled-coil domains may have significant influence upon the interactions among SNARE proteins. Since the affinity of syndet for other SNARE proteins has not been determined experimentally, in vitro binding studies of recombinant proteins will serve this purpose as well as help in defining syndet as a SNAP-25 related protein as a result of its interactions with VAMP or syntaxin. Moreover, neither of the first residues constituting the BoNT/E and BoNT/A cleavage sites of SNAP-25 are conserved in syndet, whereas in SNAP-23 the site for BoNT/E is conserved. The first residue of the cleavage site for BoNT/E in syndet
results in a conservative amino acid substitution - from Arg to Lys - of SNAP-23 to BoNT/E. It is not known whether the presence of a basic amino acid is sufficient for cleavage by for BoNT/E. The potential difference in susceptibilities of SNAP-23 and syndet to BoNT/E cleavage will be important in differentiating the functional importance of each protein in 3T3-L1 adipocytes.

One structural characteristic that is conserved between syndet and SNAP-23 is the arrangement of cysteine residues within the cysteine-rich region. SNAP-23 and syndet behave as integral membrane protein even though both proteins lack a stretch of hydrophobic residues characteristic of transmembrane region. The two proteins probably associate with the membrane via palmitoylation - a post-translational modification responsible for the tight association of SNAP-25 with the synaptosomal membrane (Hess et al., 1992). Future experiments are needed to test this hypothesis by demonstrating SNAP-23 and syndet are indeed palmitoylated.

In an attempt to detect expression of mRNA encoding SNAP-23, we have used a cDNA containing the entire coding region of human melanoma SNAP-23 as a probe for total RNA prepared from different murine tissues and cells including 3T3-L1 adipocytes as well as human melanoma. The probe only reacted positively with RNA from human melanoma. The lack of detection of SNAP-23 mRNA in murine tissues and cells with a human cDNA probe could be due to species differences. Future Northern blot analysis using lower levels of stringency for hybridization may allow detection of SNAP-23 at the nucleotide level.

The identification of SNAP-23 and syndet supports the theme of universality proposed by the SNARE hypothesis by suggesting the existence of SNAP-25 related gene products outside the nervous system. Now that new players for intracellular traffic have been identified, the next task will involve characterization of the interactions among the players as well as determining the role of each in vesicle traffic. Future knowledge in the
field of membrane traffic will provide a better understanding in mechanism underlying constitutive exocytic process which is crucial in maintaining the integrity of subcellular compartments. In addition, the information will provide insights regarding regulated exocytosis such as the insulin-stimulated docking and fusion of glucose transporter, GLUT4-containing vesicles with the plasma membrane. A better understanding of the mechanism of GLUT4 traffic will have important implication in identifying the cellular defects in diabetes mellitus, since it is generally believed that one of defects in non-insulin dependent diabetes mellitus is ineffective translocation of GLUT4 vesicle to the cell surface in response to insulin.
REFERENCES


Brennwald, P., Kearns, B., Champion, K., Keranen, S., Bankaitis, V., and Novick, P. (1994). Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. Cell 79, 245-58.


Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. Nature 345, 260-1.


Tissue Distribution of SNAP-23 and Its Subcellular Localization in 3T3-L1 Cells

Peggy P. C. Wong,* Nicholas Daneman,* Allen Volchuk,* Norman Lassam,† Michael C. Wilson,‡ Amira Klip,*1 and William S. Trimble*

*Division of Cell Biology, The Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada; †Toronto-Bayview Regional Cancer Centre, Toronto, Ontario, Canada; and ‡Department of Biochemistry, University of New Mexico, School of Medicine, Albuquerque, New Mexico

Received November 21, 1996

The SNARE hypothesis of vesicular traffic proposes that three proteins, VAMP/synaptobrevin, syntaxin, and SNAP-25, constitute a complex that docks the vesicle at the target membrane. VAMP and syntaxin isoforms have been identified outside the nervous system, and a cDNA to a SNAP-25 related protein, SNAP-23, was recently identified in human lymphocytes. Here we report the generation of isoform-specific antibodies to SNAP-23 cloned from human melanoma cells, and their use in detecting the expression and localization of the endogenous SNAP-23 protein in several tissues and cell lines. SNAP-23 was readily detected in liver, lung, kidney, and spleen, to a lesser extent in muscle and heart, and was almost undetectable in brain. The protein was also abundant in fibroblast, muscle, and fat cell lines, but relatively less enriched in neuroendocrine PC12 cells. SNAP-23 abundance did not change during differentiation of 3T3-L1 fibroblasts into adipocytes. In both, SNAP-23 was membrane-bound and below detectable levels in the cytosolic fraction. Subcellular fractionation of 3T3-L1 adipocytes revealed that the majority of the protein was associated with plasma membranes. These findings support the conclusion that a tripartite SNARE complex exists outside of the nervous system, and suggest that SNAP-23 may play a role in vesicle traffic in most cell types. © 1997 Academic Press

The binding of vesicles to, and their fusion with, target membranes is a key step in intracellular traffic in all eukaryotic cells. The SNARE hypothesis predicts that a combination of vesicular (v-SNARE) and target (t-SNARE) membrane proteins interact to form the SNARE (SNAP-receptor) complex which serves as the target for the binding and action of soluble fusion components NSF and SNAP (1). Furthermore, the specificity of vesicle targeting is thought to be mediated by the correct association of v- and t-SNARE isoforms. In the nervous system the vesicle-associated membrane protein VAMP acts as a v-SNARE and binds to the t-SNARE membrane proteins syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa) to form a stable tripartite complex. Multiple isoforms of VAMP and syntaxin have been identified, and several of these are known to be expressed outside the nervous system. However, until very recently, only two alternatively spliced forms of SNAP-25 were known to exist, and their expression was found to be restricted to the nervous system (2, 3) and neuroendocrine cells such as pancreatic islets of Langerhans (4-6), adrenal chromaffin cells (7), and anterior pituitary cells (8). If the basic tenets of the SNARE hypothesis are correct, and if this ternary cassette of proteins also functions to mediate membrane fusion in other cell types, then SNAP-25-like molecules should be present in non-neuronal cells undergoing regulated membrane traffic events.

Recently, Ravichandran et al. (9) described the cDNA cloning of a novel isoform of SNAP-25, named SNAP-23, and observed expression of its mRNA in several non-neuronal tissues. To examine the presence and distribution of SNAP-23 at the protein level, we have isolated a full length cDNA clone from human melanoma cDNA library and produced antibodies specific for SNAP-23. Using these reagents we demonstrate the presence of this protein in a variety of tissues and cell lines. In contrast, we were unable to detect SNAP-25 outside neuronal/neuroendocrine cells. Further, we show that SNAP-23 is localized at the plasma membrane in the 3T3-L1 adipocyte cell line, where it may likely act as a t-SNARE along with syntaxin 4. These results support the generality of the SNARE hypothesis and suggest that the v-/t-SNAREs ternary complex may be found in most cell types.

1To whom correspondence should be addressed. Fax: (416) 813-5028.
MATERIAL AND METHODS

Materials. IMAGE Consortium expressed-sequence tag (EST) cDNA #384468 (GenBank™ accession number H82169) was obtained from Research Genetics. SNAP-25 monoclonal antibody SMI 81 (here called aSN25mAb) was obtained from Sternberger Monoclonals Inc. Anti α1 Na⁺/K⁺ ATPase monoclonal antibody 61H was kindly provided by Dr. M. Caplan (Yale University, New Haven, CT). GLUT4 polyclonal antibody was from East Acres Biologicals. An affinity purified antisera (aSN25pAb) was raised against residues 93-206 of SNAP-25 isoform (10).

Cloning and bacterial expression of SNAP-23 and SNAP-25. An EST clone (see Results) labeled with [α-32P]dCTP was used to screen approximately 500,000 plaques from a UNI-ZAP XR human melanoma cDNA library. Twelve positive plaques were identified and their cDNA inserts were subcloned into Bluescript plasmids (Stratagene) for single-stranded DNA sequencing using the Sequenase version 2.0 kit (United States Biochemical). Sequences of the 5' end of the twelve clones revealed one which began at nucleotide 50, 43 base pairs upstream from the putative initiation codon reported for the recently published SNAP-23 cDNA (9). The coding sequence of this clone was amplified by polymerase chain reaction (PCR) using primers (nucleotides 93-110, 714-731) which flanked the coding region. This amplified fragment was then subcloned into pGEX-2T vector. The entire sequence was confirmed by automated sequence analysis (Biotechnology Service Centre, Toronto, ON) and found to be identical to human SNAP-23 cDNA (9). GST fusion proteins of SNAP-23 and SNAP-25 were expressed and purified from Escherichia coli (11). The cDNA insert of the EST clone was isolated and subcloned into the pET-32a expression vector (Novagen).

Generation of SNAP-23 antisera. Two rabbit antisera specific to SNAP-23 were raised. Antibody aSN23.C116 was produced against a recombinant protein encoding carboxy-terminal 116 residues of SNAP-23 (amino acids 96-211) that was liberated from thioredoxin by thrombin cleavage. The serum was affinity purified on a column of Affigel 15 to which the GST SNAP-23 fusion protein described above was coupled. Antibody aSN23.C12 was raised against a peptide corresponding to carboxy-terminal residues 200-211 of SNAP-23 (H2N-1ANARAKKLDSC-OH) coupled to keyhole limpet hemocyanin (Biotechnology Service Centre, Toronto, ON). An affinity column was produced by coupling the same peptide to SulfoLink Gel (Pierce Chemical Company). Antibodies bound to affinity columns were eluted with 20 mM glycine and 0.5 M NaCl, pH 2.5 and the eluates were neutralized with 0.1 M Tris, pH 8.5.

Cell culture. The following cell lines were grown: rat adrenal pheochromocytoma PC12, murine melanocyte melan-b, and human melanoma MelW. Myoblasts of the rat muscle cell line, L6, were allowed to fuse and differentiate into myotubes upon fusion (12, 13). Mouse 3T3-L1 fibroblasts were differentiated into adipocytes as described previously (14).

Membrane isolation and immunoblotting. Total membranes (TM) were prepared from confluent cells. All procedures were carried out at 4°C. Monolayers were washed with homogenization buffer (20 mM Hepes, 255 mM sucrose, and 1 mM EDTA, pH 7.4), scraped with a rubber policeman in homogenization buffer containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, and 10 μM E-64) and homogenized with 10 strokes using a cell cracker. Homogenates were centrifuged at 1,000 x g for 3 min. The resultant postnuclear supernatants were centrifuged at 245,000 x g for 90 min to yield total membranes. The supernatants represented the cytosolic fraction. Confluent 3T3-L1 adipocytes (7-10 days post-differentiation) were sub-fractionated as described previously. Organs were dissected from mice and microsomes were prepared according to the protocol described (15). Protein samples were solubilized in Laemmli sample buffer (16) and separated by SDS-PAGE (17). Proteins were detected with the following primary antibodies: anti α1 Na⁺/K⁺ ATPase monoclonal (1:250), anti SNAP-25 monoclonal aSN25mAb (SMI 81, 1:2000), anti GLUT4 polyclonal (1:1000), anti SNAP-25 polyclonal (aSN25pAb, 1:200), affinity-purified SNAP-23 polyclonal antisera aSN23.C116 and aSN23.C12 were used at 4 ng/ml and 0.12 mg/ml respectively. aSN25pAb was preincubated with bacterial GST protein followed by incubation with glutathione linked to agarose beads to remove any antibodies that may cross-react with the GST portion of SNAP-23 or SNAP-25 fusion proteins before immunoblotting. Enhanced chemiluminescence was used as the method for detection.

RESULTS AND DISCUSSION

In preliminary experiments using a full-length SNAP-25 cDNA probe, we were unable to detect signals corresponding to SNAP-25 in total RNA from 3T3-L1 fibroblasts or adipocytes under high or low stringent conditions (18). In addition, immunoblotting of proteins from 3T3-L1 adipocytes with the monoclonal antibody specific to SNAP-25 (aSN25mAb) also failed to give a positive result. However, when plasma membranes prepared from 3T3-L1 adipocytes were probed with a polyclonal SNAP-25 antiserum (aSN25pAb) raised to near full-length of the protein (10), a polypeptide that migrated at 29 kDa was detected. The detection was specific since it was prevented upon preincubation of the antiserum with recombinant SNAP-25 protein (18). These results implied that an immunoreactive species related to SNAP-25 existed in 3T3-L1 cells and prompted us to search for such a gene product.

Through a search of the Genbank™ database we identified a human EST clone which encoded a protein predicted to share 64% sequence identity with the C-terminal 78 amino acids of SNAP-25. Using this cDNA as probe, we screened approximately 500,000 plaques of a human melanoma cDNA library. Twelve positive plaques were isolated, the longest of which contained a cDNA of approximately 2.0 Kb. Sequence analysis of this clone revealed that it was identical to the recently described SNAP-25-related gene product SNAP-23 (9), and contained the entire coding sequence.

We have generated two antisera to the C-terminal portion of SNAP-23. Antibody aSN23.C116 was raised against a truncated portion of the protein (amino acids 96-211) and antibody aSN23.C12 was directed against a peptide corresponding to residues 200-211 of SNAP-23. Equal amounts (1 μg) of GST SNAP-23 and GST SNAP-25 fusion proteins were immunoblotted to test the specificity of the two affinity-purified antisera. Both of these antisera detected only SNAP-23 fusion protein but not SNAP-25 (Fig. 1, top two panels). Moreover, no cross-reactivity to the GST portion of the recombinant proteins was detected and no immunoreactivity was detected with the preimmune sera (data not shown). Further characterization of the two antibodies used in preliminary studies (18) is shown in the bottom two panels of Fig. 1. The polyclonal SNAP-25 antiserum, aSN25pAb, cross-reacts to both SNAP-23 and SNAP-25, whereas the monoclonal SNAP-25 antibody, aSN2-
bating the antiserum with excess
in PC12 neuroendocrine cells (Fig.
various
the immunoreactive
human
cel1
distribution of SNAP-23, SNAP-25 showed a
(Fig. 2A, rniddle panel). In contrast to the
3T3-L1
nous expression of the
SNAP-23
detected
3mAb,
(9).
5mAb, is specific for SNAP-25. These data indicate that
the immunoreactive protein detected by αSN25pAb in
3T3-L1 adipocytes in previous studies was most likely
SNAP-23 and not SNAP-25 (18).

The predicted amino acid sequence of SNAP-23 is
59% identical and 72% similar to SNAP-25b isoform
(Northern blot analysis demonstrates a wide tissue
distribution of SNAP-23 mRNA (9), however endoge-
uous expression of the protein has not been examined.
Here we report the expression of SNAP-23 protein in
various cell lines and tissues. SNAP-23 protein was
detected using αSN23.C12 in both non-neuroendocrine
cell lines such as mouse 3T3-L1 fibroblasts, 3T3-L1 adip-
cocytes, melan-b melanocytes, rat L6 myotubes, and
human melanoma MeWo cells as well as lower levels
in PC12 neuroendocrine cells (Fig. 2A, top panel). The
protein detected by the αSN23.C12 antiserum migrates
at approximately 29 kDa. The immunodetection of
SNAP-23 was specific since it was blocked by preincu-
bating the antiserum with excess amount of the peptide
(Fig. 2A, middle panel). In contrast to the widespread
distribution of SNAP-23, SNAP-25 showed a very re-
stricted expression, limited only to the neuroendocrine
PC12 cells (Fig. 2A, bottom panel).

In contrast to our inability to detect SNAP-25 in 3T3-
L1 adipocytes and L6 muscle cells, Jagadish et al. (19)
recently reported the expression of SNAP-25a in fat
tissue and SNAP-25b in skeletal muscle. The detection
of SNAP-25 in the fat and muscle tissues could poten-
tially result from the presence of nerve terminals in
the biological material. Alternatively, the amount of
SNAP-25 protein in our fat and muscle cell lines may
be below the detection level of our antibodies.

As shown above for cell lines, SNAP-23 was also
widely distributed in different tissues from rats and
mice (Fig. 2B). The protein was found to be abundant
in lung, liver, spleen and kidney, but relatively less
concentrated in rat skeletal muscle and mouse heart.
Upon longer exposure of the immunoblot to autoradiog-
raphy, a low level of SNAP-25 could also be detected
in the mouse brain (result not shown).

The subcellular distribution of SNAP-23 is not
known but, like SNAP-25, it lacks a stretch of hy-
drophobic residues necessary to form a transmembrane
domain. In the case of SNAP-25, the majority of the
protein is tightly associated with synaptosomal mem-
brates (20) probably via palmitoylation of one or more
of the four cysteine residues clustered in the middle of
the protein (21). It has recently been shown that dele-
tion of a stretch of 12 amino acids encompassing the 4
cysteine residues renders the deletion mutant of
SNAP-25 completely soluble and resistant to [3H] pal-

![FIG. 1. Characterization of SNAP-23 and SNAP-25 antibodies. Immunoblot analysis was used to test the specificity of four antibodies to either SNAP-23 or SNAP-25 GST fusion proteins. One microgram of each fusion protein was subjected to SDS-PAGE and immunobotted with the following antibodies: αSN23.C116 affinity purified antiserum raised to the C-terminal 116 amino acids of SNAP-23; αSN23.C12 affinity purified antiserum raised to the C-terminal 12 amino acids of SNAP-23; αSN25pAb polyclonal antiserum raised to residues 33-206 of SNAP-25; and αSN25mAb (SMI 81) monoclonal antibody to SNAP-25.]

<table>
<thead>
<tr>
<th>kDa</th>
<th>PC12</th>
<th>L6m</th>
<th>L6m F</th>
<th>3T3-L1 A</th>
<th>Melan-b</th>
<th>MeWo</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>αSN23.C12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>αSN23.C12 + SNAP-23 peptide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>αSN25mAb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![FIG. 2. Expression of SNAP-23 in cell lines and tissues. (A) Immunoblot of total membranes prepared from various cell lines of different species. Twenty micrograms of proteins from rat neuroendo-
crine PC12 cells, rat L6 myotubes (L6 Mt), mouse 3T3-L1 fibroblasts (3T3-L1 F) and 3T3-L1 adipocytes (3T3-L1 A), mouse melanocytes (melan-b), and human MeWo melanoma cell line were resolved by
SDS-PAGE and probed for SNAP-23 with αSN23.C12 antiserum
(0.12 μg/ml) (top). The specificity of immunodetection was confirmed
by preincubation of αSN23.C12 antiserum with excess amount of
peptide (0.2 μg/ml) (middle). Detection of SNAP-25 by the monoclonal
antibody αSN25mAb (SMI 81) was restricted to neuroendocrine
PC12 cells (bottom). (B) Immunoblotting of total membranes from
rat skeletal muscle (Sk. muscle) and other tissues from mouse with
αSN23.C12 antiserum. |
mitic acid labeling (22). In order to determine the localization of SNAP-23 in 3T3-L1 fibroblasts we used subcellular fractionation. The results in Fig. 3A show that SNAP-23 is exclusively found in the membrane fraction (TM) with no detectable amount found in the cytosolic fraction (CTY). Since SNAP-23 also contains a cluster of cysteine residues, it may associate with the membrane by a similar mechanism. Interestingly, SNAP-23 contains 5 cysteine residues in this cluster, as is the case for Torpedo SNAP-25 (23). In contrast, the mammalian SNAP-25a and SNAP-25b have each a substitution of one of the cysteines, breaking the cluster arrangement. The extra cysteine residue found in SNAP-23 and Torpedo SNAP-25 may define an ability to associate with specific proteins.

3T3-L1 fibroblasts can be differentiated into adipocytes, hence allowing us to study the protein at two stages of differentiation. Proteins that are involved in intracellular traffic have been shown to be expressed at different levels following differentiation into adipocytes. For example, cellubrevin, a member of the synaptotubrevin family, is expressed predominantly in differentiated 3T3-L1 adipocytes (24). In contrast, syntaxin 4 is already abundant at the fibroblast stage (Volchuk and Klip, unpublished). As can be seen in Fig. 3B, there was no significant change in the level of expression of SNAP-23 upon differentiation, suggesting that the protein is necessary in both fibroblasts and adipocytes.

The detection of SNAP-23 in 3T3-L1 adipocytes provided us with an opportunity to further study the subcellular distribution of the protein in plasma membrane (PM), light density microsomes (LDM), and cytosol (CTY). In contrast to fibroblasts, the fractionation protocol for 3T3-L1 adipocytes has been well established (25). The α1 subunit of Na⁺/K⁺ ATPase is a useful marker for the PM. This protein was detected almost exclusively in the PM and not in the LDM or CTY of 3T3-L1 adipocytes (top panel of Fig. 3C). The LDM contains the majority of the GLUT4 glucose transporter which locates intracellularly in unstimulated cells (Fig. 3C, middle panel). The majority of SNAP-23 was found to be associated with the PM (Fig. 3C, bottom panel). Occasionally, a small amount of SNAP-23 was also detected in the LDM (not shown).

The functional importance of SNAP-25 in synaptic vesicle traffic has been established by the discovery that it is the substrate for botulinum neurotoxins A and E (26, 27). These toxins are endopeptidases and potent inhibitors of neurotransmitter release. Botulinum neurotoxins A and E cleave SNAP-25 at distinct peptide bonds (10). Sequence alignment of SNAP-23 and SNAP-25 proteins reveals that the two amino acids which constitute the proteolytic cleavage site for botulinum neurotoxin type E, but not those for type A, are conserved in SNAP-23. Therefore we predict that SNAP-23 may be protected from proteolytic cleavage by botulinum neurotoxin A. Botulinum toxin E may be a useful tool to discern whether SNAP-23 plays a role in exocytosis. Indeed, we have shown that both SNAP-23 and SNAP-25 are present in PC12 cells; in these cells, exocytosis of large dense core vesicles is inhibited completely by botulinum neurotoxin E but not A, although both toxins effectively cleaved SNAP-25 (28). The presence of SNAP-23 and its potential insensitivity to proteolysis by botulinum neurotoxin A may explain the incomplete inhibition in the peptide hormone secretion observed in PC12 cells.

The detection of SNAP-23 in non-neuronal cells completes the tenet of the SNARE hypothesis, that functional homologues of SNAP-25, syntaxin-1 and VAMP exist at all steps of vesicle docking/fusion in non-neuronal cells. Since muscle and 3T3-L1 cells express syntaxin 4 (29, 30) and VAMP-2 (31), it is tempting to speculate that SNAP-23 could potentiate the binding of syntaxin 4 to VAMP-2 and in this way contribute to the formation of a stable complex that would facilitate exocytic activity in these tissues.

ACKNOWLEDGMENTS

We thank Dr. Michael Caplan for the anti-Na⁺/K⁺-ATPase antibody, Dr. Z. Liu for help in the affinity purification of antisera, and...
REFERENCES

SNAP-23 IS LOCATED IN THE BASOLATERAL PLASMA MEMBRANE OF RAT PANCREATIC ACINAR CELLS

Herbert Y. Gaisano¹, Laura Sheu¹, Peggy P. C. Wong², Amira Klip²,³, William S. Trimble²,³.

From the Departments of Medicine¹, Biochemistry² and Physiology³, University of Toronto, The Toronto Hospital, Toronto, Ontario M5S 1A8¹, and the Division of Cell Biology of the Hospital for Sick Children Research Institute, Toronto, Ontario, M5G 1X8².

Please address correspondence to:
Herbert Y. Gaisano
Room 7226 Medical Sciences Building
University of Toronto
Toronto, Ontario, Canada M5S 1A8
Tel. (416) 978-1526
Fax. (416) 978-8765
E-mail. herbert.gaisano@utoronto.ca

Running title: SNAP-23 in rat pancreatic acinar cells
The SNARE hypothesis proposes that specificity of exocytotic fusion processes is regulated by the appropriate interactions of the vesicle (v-) SNARE VAMP/synaptobrevin and the target membrane (t-) SNAREs syntaxin and SNAP-25. Recently we demonstrated that VAMP-2 was present on the pancreatic acinar zymogen granule membrane while syntaxin-2, -4 and -3 were localized to the apical plasma membrane, basolateral membrane and zymogen granule membrane, respectively. However, other studies had suggested that SNAP-25 was not expressed in non-neuronal cells such as the acinar cell. We therefore began to search for SNAP-25 like proteins in exocrine pancreatic tissue. Using an isoform-specific antibody generated to the first novel SNAP-25-like protein reported outside the nervous system, SNAP-23, we now show by immunoblotting that acinar cells express SNAP-23, and this t-SNARE is located on zymogen granule membranes and the plasma membrane. Using laser confocal immunofluorescence microscopy we show that the plasma membrane component of SNAP-23 is distinctly localized to the basolateral plasma membrane of acinar cells. This colocalization of t-SNAREs SNAP-23 and syntaxin-4 to the basolateral plasma membrane, coupled with their ability to form a ternary complex with the v-SNARE, VAMP-2, suggest that this SNARE complex may serve as the molecular substrate for basolateral membrane fusion of secretory granules, currently believed to be a phenomenon occurring in acute pancreatitis.

Key words: exocytosis / pancreatitis / SNAREs
The pancreatic acinar cell has long been used as a model cell type for the study of exocytosis in non-excitable cells (1). Through intensive study, the kinetics and morphology of complex constitutive and regulated exocytotic pathways have been defined in these highly polarized cells (2). Regulated exocytosis of zymogen granules (ZG) in response to low, physiologic concentrations of secretagogues, occurs at a limited apical portion of the acinar cell, which constitutes only 5-10% of the total PM surface (1,3). Enzymatic ZG contents are then delivered to the collecting ducts of the pancreas and subsequently emptied into the intestinal lumen to digest nutrients. In contrast, exposure of acinar cells to supraphysiologic secretagogue concentrations either in vitro or in vivo appears to cause a blockade of apical exocytosis, and aberrant ZG fusion events including intragranular fusions and fusion with the basolateral plasma membrane (4). In vivo, this basolateral ZG secretion results in experimental conditions which appear analogous to clinical pancreatitis (4) where serum levels of pancreatic enzymes are elevated. However, the mechanisms controlling normal exocrine secretion, and their possible dysfunction during pancreatitis, remain to be determined.

Recent studies by us and others suggest that regulation of ZG fusion may follow the same basic principles as described in the SNARE hypothesis. This general model was derived to explain mechanisms controlling neurotransmitter release, and extrapolated to cover most membrane transport processes in virtually all cells (5). The SNARE hypothesis predicts that cytosolic NEM-sensitive factors (NSF) and soluble NSF attachment proteins (SNAPs), bind to
(t-SNAREs), to mediate the docking and fusion of the two membranes (5). In the nervous system the vesicle-associated membrane proteins (VAMPs) on the synaptic vesicle, along with syntaxin 1 and synaptosomal-associated membrane protein of 25kDa (SNAP-25) on the synaptic plasma membrane, forms a stable complex which acts as the receptor (SNARE) for the soluble factors (reviewed in 6). It is hypothesized that specific v- and t-SNARE combinations may control the accuracy of vesicle-target membrane interactions. The presence of multiple isoforms of VAMP (7) and syntaxin proteins (8), and the specificity of their interactions (9), support this tenet. However, SNAP-25 is expressed predominantly in the nervous system and in neuroendocrine cells (10) leaving non-neural cells without a critical SNARE component. Recently, Ravichandran et al. (11) reported the cDNA cloning of a novel isoform of SNAP-25, called SNAP-23, which is broadly expressed in non-neuronal tissues, including the pancreas. Antibodies specific for SNAP-23 detect a single protein species associated with the plasma membrane which is abundant in most non-neural but not neural cells (12). This identification provides a complete cassette of SNARE proteins in virtually all cell types.

To determine if exocrine granule secretion is controlled by mechanisms similar to those involved in neurotransmitter release, we began to search for expression of SNARE homologs in pancreatic acinar cells. Initially we demonstrated that VAMP isoform-2 was an integral component of the zymogen granule membrane and cleavage of this protein by tetanus toxin resulted in inhibition of calcium-evoked digestive enzyme secretion (13,14). More recently we have demonstrated that acinar cells express four isoforms of syntaxin with syntaxin isoform-2
4 on the basolateral plasma membrane of the pancreatic acinar cell (15). Taken in the context of
the SNARE hypothesis, their restricted appearance at these membrane compartments indicates that
each of these syntaxins may serve as a distinct exocytotic target for each site (6).

In the present study, we demonstrate that acinar cells also express SNAP-23. Using an
affinity purified antibody directed to the C-terminus of SNAP-23 (12), we show that SNAP-23,
but not SNAP-25, can be detected in the acinar cells while endocrine cells of the pancreas express
SNAP-25 only. We also show that SNAP-23, like syntaxin-4, is specifically localized to the
basolateral plasma membrane of the acinar cell. The basolateral location of SNAP-23, along with
that of syntaxin 4, suggests that these proteins do not participate in apical secretion, but they may
be involved in normal or aberrant secretory events occurring at the basolateral surface.
Antibody Generation. Rabbit polyclonal antibodies to VAMP-2, syntaxins-2 through -4 and SNAP-23 were generated, subjected to affinity purification and their specificity validated as we have previously reported (12-15). Anti-SNAP-23 specific antibody was generated against a peptide corresponding to the carboxy-terminal residues 200-211 of SNAP 23, called αSN23.C12, and affinity purified on columns to which the peptide had been coupled (12). Antibody raised against SNAP-25, kindly provided by M.K. Bennett, was generated against the bacterially-expressed full length fusion protein of SNAP-25 and affinity-purified using full-length fusion protein immobilized on Affigel beads (Bio-Rad, Richmond, CA). The specificity of these antibodies was determined by immunoblotting against the recombinant full-length fusion proteins and against native tissues (see Figures 1 through 3) which showed that while the SNAP-23 antibody is specific only for SNAP-23, the SNAP-25 antibody cross-reacts with both SNAP-23 and SNAP-25.

Subcellular Membrane Preparations and Immunoblotting. Pancreatic acinar ZG membranes and plasma membranes were prepared from excised pancreata of Sprague-Dawley rats (250-300 g) made diabetic, as described (13-15), by treatment with streptozotocin which obliterates β-cells. Total membranes were prepared from 3T3-L1 fibroblasts as previously reported (12). SDS-PAGE and immunoblots were performed as described (13) using primary antibodies diluted to 1:1000. Detection of the antigen on the blot was by enhanced chemiluminescence (ECL, Amersham Corp.). Specificity was determined by preincubating the antibodies with 10 M excess
temperature prior to use as in Figure 1.

Immunofluorescence Confocal Microscopy. These studies were performed as previously described (14,15). Briefly, pancreata from a diabetic or normal rat that have been perfused in vivo with 4% paraformaldehyde in physiological saline and frozen in liquid nitrogen, were embedded in Tissue Tek OCT. 5-μm cryostat sections were generated, rinsed in PBS and postfixed in 4% paraformaldehyde, rinsed again with PBS, and then blocked with 5% normal goat serum with 0.1% saponin for 30 min. The tissue on the glass slides was then incubated at room temperature for 1 hr with the primary antibodies: rabbit anti-SNAP-23 (1:50), rabbit anti-SNAP-25 (1:50), guinea pig anti-insulin (1:100, a gift from R. A. Pedersen, University of British Columbia, Vancouver, Canada). Specificity was determined by preincubation of the primary antibody with 10 M excess (~20 μg/ml) of full length recombinant fusion proteins of the appropriate isoforms for 1 hr at room temperature prior to use. These were then rinsed with 0.1% saponin, and then treated with appropriate FITC- or rhodamine-labeled secondary antibody (1 hr, room temperature): goat anti-rabbit (1:500) and goat anti-guinea pig (1:500). Phalloidin conjugated to FITC (Molecular Probes, Eugene, OR) was added during the secondary antibody step at 1 U/slide. The slides were then mounted with fading retarder; 0.1% p-Phenylenediamine in glycerol, and examined using a laser scanning confocal imaging system (Carl Zeiss, Thornwood, NY).
SNAP-23 is present on pancreatic plasma membrane and zymogen granule membranes.

To determine the presence and subcellular distribution of SNAP-23 and/or SNAP-25 immunoreactive proteins in the pancreatic acinar cell, we isolated preparations of highly purified pancreatic acinar ZGM and PM for immunoblot analyses (Figure 1). To avoid possible contamination with islet membranes which we (16) and others (17) have reported to be abundant in SNAP-25, membranes were isolated from streptozotocin-treated rats as previously described (13-15). These were then compared to a homogenate of rat brain, which is abundant in SNAP-25 (18), and 3T3-L1 fibroblast total membranes, which are abundant in SNAP-23 (12). The top row shows that SNAP-23, which migrated at about 30kDa, is abundant in both pancreatic PMs and ZGMs as in the 3T3-L1 fibroblast membranes, but is virtually absent in the rat brain. The SNAP-23 signal was not affected by preincubation with excess GST-SNAP-25 (1 μg) (second row) but was blocked when preincubated with GST SNAP-23 (third row), confirming the specificity of the SNAP-23 antibody. The SNAP-25 antibody gave a strong immunoreactive signal in the rat brain and a weaker signal in the pancreatic PM, ZGM and 3T3-L1 membranes (fourth row). GST-SNAP-23 had no effect on the SNAP-25 signal in rat brain but significantly inhibited the signal in the pancreatic membranes and 3T3-L1 membranes (row 5), whereas GST SNAP-25 completely blocked all of the signals from this antibody (row 6). Furthermore, a monoclonal antibody (SMI 81, Sternberger Monoclonal Inc.) which recognizes brain and islet SNAP-25 failed to recognize the acinar isoforms. However, this antibody gave a strong cross-reaction to high molecular weight material within the ZG (data not shown) and was not used
pancreatic PM and ZGM, and 3T3-L1 membranes, was actually SNAP-23 to which the SNAP-25 antibody cross-reacted.

The SNAP-23 on the ZGM is tightly associated with that membrane since it was resistant to washing with NaBr (0.25 M, pH 11) which removes peripherally adhering proteins. It is unlikely that the ZGM signal could be due to contamination with PMs since the ZGMs were prepared from a highly purified ZG fraction (13-15), and the Coomassie blue staining of the ZGM and PM fractions showed the membrane protein profiles to be distinct (data not shown). Furthermore, a slight contamination with PM would not explain the intensity of the ZGM signal which is equivalent to that on the PM.

**SNAP-23 and SNAP-25 are present in pancreatic acinar cells and islets, respectively.** The pancreas provides an ideal opportunity to compare the endocrine and exocrine tissues side-by-side for the presence of SNAP-25 and SNAP-23 (Figure 2). We therefore performed double label immunofluorescence microscopy on pancreatic sections taken from normal (non-diabetic) rats using affinity-purified SNAP-23 and SNAP-25 antibodies individually along with antibodies specific to rat insulin raised in guinea pigs. In the islets, insulin (Figure 2b) was restricted to cells in which SNAP-25 was abundant and localized to the cell membrane (Figure 2a). In contrast, SNAP-23 antibody strongly stained the plasma membrane of every acinar cell (Figure 2c), but minimally if at all in the insulin-positive islets (Figure 2d). Therefore, unlike VAMP and syntaxin proteins which are expressed in both the endocrine and exocrine pancreas (14-16),
SNAP-23 is located on the basolateral plasma membrane of the pancreatic acinar cell.

To more precisely map the subcellular location of SNAP-23 in acinar cells we have performed double label immunofluorescence microscopy on sections from the pancreas of diabetic rats with the SNAP-25 and SNAP-23 antibodies along with phalloidin, which labels actin filaments near the apical surface of the acinar cell (15,19). As shown in Figure 3a, the SNAP-23 antibody labels the basal and lateral plasma membrane of every acinar cell but not the apical portions which are labeled by phalloidin (Figure 3b). Anti-SNAP-23 also gave weak and inconsistent labelling of internal structures which may include the ZGs or other intracellular membranes. The SNAP-23 signal was completely blocked by GST-SNAP-23 (Figure 3c) but was not affected by preincubation of the antibody with GST-SNAP-25 (Figure 3d). A similar pattern was detected with the SNAP-25 antibody (Figure 3e) when the laser intensity was significantly increased, although this could not be seen at intensities used for SNAP-23 (See Figure 2a). The SNAP-25 signal on the acinar cell was completely blocked by preincubation of the antibody with GST-SNAP-25 (not shown), as well as with GST-SNAP-23 (Figure 3f). The GST-SNAP-23, however, did not block the SNAP-25 signal of a nerve fiber straddling the basal surface of an acinus (arrowhead in f). Again, this confirms that the only immunoreactivity detected by the SNAP-25 antibody in exocrine acinar cells was SNAP-23. The ZG membrane SNAP-23 signal in our immunofluorescence study was not consistently seen, in contrast to our immunoblotting study. This may be due to preferential sensitivity of the ZGM-bound SNAP-23 to the aldehyde fixatives.
SNAP-23 is a recently described, widely expressed isoform of the neuronal t-SNARE SNAP-25 (11,12). SNAP-23 is 59% identical to SNAP-25 at the amino acid level and its mRNA and protein are expressed in non-neuronal tissues (11,12). To determine which of these two proteins are present in pancreatic acinar cells, we used polyclonal antibodies specific for SNAP-25 (15,16), and SNAP-23 (12) in Western blotting and immunocytochemical studies. The antibodies raised against SNAP-25 recognized proteins in the rat brain, pancreatic islet, and exocrine acinar cell. However, the latter signal appears to be the result of cross-reaction since it could be fully blocked by pre-incubation of the antibodies with recombinant SNAP-23 proteins. In contrast, anti-SNAP-23 antibodies gave strong signals for acinar cell membrane preparations and little if any signal for neural membranes, and this signal was not sensitive to blocking by preincubation with GST-SNAP-25 but was eliminated by GST-SNAP-23. Together with a previous study which failed to detect SNAP-25 in the acinar cell using an antibody specific to the carboxyl terminus of SNAP-25 (17), our results show that SNAP-23 is the predominant isoform and that SNAP-25 is not expressed in these cells.

The pancreas is a tissue which allows simultaneous assessment of protein expression in both endocrine and exocrine cells. We observed that SNAP-23 was expressed in the exocrine but not endocrine cells, the reciprocal of that seen for SNAP-25. Interestingly, the other SNARE proteins VAMP-2 and syntaxin-1 appear to be expressed in both cell types (13,15-17) albeit at much higher levels in endocrine cells. This suggests that the SNAP-23/SNAP-25 component of
of this difference will await further characterization of the precise role of SNAP-23 in membrane fusion.

Within the pancreatic acinar cell, SNAP-23 is present in abundance on the PM and on the ZGM. On the PM, the SNAP-23 was highly enriched on the basolateral membranes in the acinar cells and was excluded from the apical surface. These subcellular locations overlap with the patterns we previously obtained for syntaxin-3 (ZGM) and syntaxin-4 (basolateral PM) in acinar cells (15). Although SNAP-23 can form binary interactions with all four of the syntaxin isoforms in vitro, it is interesting to see that it co-localizes with only these two, and that no co-localization was seen with the apically localized syntaxin-2. SNAP-23 is therefore unlikely to be localized solely by its association with the syntaxins, but whether it is targeted by association with other proteins or carries its own targeting signals is not known.

The appearance of SNAP-23 at the basolateral surface represents the first evidence for regional localization of this new t-SNARE isoform within polarized cells and argues that this protein may participate, along with syntaxin-4 and a member of the VAMP family to mediate basolateral membrane fusion events. In vitro studies have shown that VAMP-1 and VAMP-2 can bind to syntaxin-1 or syntaxin-4 with high affinity, but not with syntaxin-2 or -3, suggesting specificity of vSNARE-tSNARE interactions (9). SNAP-25 and SNAP-23, on the other hand, do not appear to contribute to this specificity and are capable of binding to each of the syntaxin and VAMP isoforms (9,11). Given the presence of VAMP-2 on the ZGM, a complete cassette
rarely occurs under normal circumstances, this mechanism must be under additional levels of control. Interestingly, in response to high agonist concentrations, interstitial pancreatitis can result from the inappropriate release of zymogens to the basolateral surfaces (4,20). Whether such events result from a failure of such control mechanisms, or entry of the vesicles into minor constitutive exocytosis pathways operating through the basolateral surfaces (21) is not known.

The functional significance of SNAP-23 localized to the ZGM is not clear, but vesicular localizations have been previously detected for both syntaxin-1 and SNAP-25 on synaptic vesicles (22) and on the chromaffin granule membrane (23,24). Within the context of the SNARE hypothesis, ZGM localized SNAP-23 could be explained if the ZGM itself were to act as a target membrane for fusion (15). This idea is supported by previous studies demonstrating ZG-ZG fusions in vivo (4) and in vitro (25). Such fusions between Golgi-derived progranules may be necessary for the formation of immature and mature ZGs (26), while fusions between mature granules could be important to promote compound exocytosis. If SNAP-23 forms SNARE complexes with syntaxin-3, the v-SNARE with which they associate remains to be identified.

Finally, the location of SNAP-23 to the basolateral membranes, coupled with the observation that syntaxin isoforms were also distinctly localized within these cells, suggests that apical secretion is quite distinct from basolateral secretion. Since the only identified v-SNARE on the ZGM, VAMP-2, does not form stable complexes with the apical t-SNARE syntaxin-2 in vitro, it is possible that additional isoforms of SNAP-25 and VAMP may exist to provide a
secretion may occur by means other than those explained in the SNARE hypothesis. It has been shown in Madin-Darby canine kidney cells that vesicular transport from the trans-Golgi network to the basolateral plasma membrane is dependent on NSF and αSNAP, and is sensitive to tetanus and botulinum neurotoxins which cleave VAMP-2 (27). In contrast, apical secretion in MDCK cells is independent of NSF, αSNAP and insensitive to tetanus toxin (27). In our studies, we have found that while Ca²⁺-evoked amylase secretion from isolated, permeabilized acinar cells can be inhibited by tetanus toxin cleavage of VAMP-2, this inhibition is always partial (13). We are currently investigating the possibility that the permeabilized preparation may exhibit both apical and basolateral secretion with only the latter being sensitive to tetanus toxin. If so, this may provide a valuable model system to examine the mechanisms controlling normal granule targeting specificity and to develop compounds which could be effective at blocking the basolateral secretion associated with pancreatitis.

In summary, we have demonstrated that SNAP-23 is the predominant isoform of the SNAP-25 t-SNARE family expressed in the exocrine cells of the pancreas. Furthermore, we have demonstrated that it is localized abundantly on the basolateral surface of these cells where it could interact with syntaxin-4 and the ZGM v-SNARE VAMP-2 to participate in the aberrant secretory events associated with experimental pancreatitis. More work is required to identify the apical and vesicular proteins responsible for regulated apical secretion in acinar cells.
supplying the anti-SNAP-25 and anti-insulin antibodies, respectively. This work was supported by grants from the Medical Research Council of Canada to WST and HYG, the Fraser Elliott Foundation to HYG, and the Juvenile Diabetes Foundation to AK and WST. HYG is a recipient of an American Gastroenterology Association Foundation/Industry Research Scholar Award.

Footnotes.

1The abbreviations used are: VAMP-2, vesicle associated membrane protein isoform 2; ZG, zymogen granule; ZGM, zymogen granule membrane(s); SNAP-25 (and 23), synaptosomal associated protein of 25 kDa (and 23 kDa); PM, plasma membrane(s); SNARE, soluble NSF-attachment protein (SNAP) receptor; v-SNARE, vesicle-SNARE; t-SNARE, target-SNARE; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.


SNAP-23 is present on pancreatic acinar plasma membranes and zymogen granule membranes. Highly purified pancreatic acinar plasma membranes (PM, 10 μg of protein/lane) and zymogen granule membranes (ZGM, 10 μg of protein/lane) from diabetic (streptozotocin-treated) rats, crude brain homogenates (rat brain, 5 μg of protein/lane) and 3T3-L1 fibroblast microsomes (3T3-L1, 10 μg of protein/lane) were prepared, electrophoresed on a 15% SDS-polyacrylamide gel and blotted on Immobilon-P transfer membranes (Millipore) as described in Methods. These were the immunoblotted with: Top row - SNAP-23 antibody; second row - SNAP-23 antibody preincubated with GST-SNAP-25 (1 μg); third row - SNAP-23 antibody preincubated with GST-SNAP-23 (1 μg); fourth row - SNAP-25 antibody; fifth row - SNAP-25 antibody preincubated with GST-SNAP-23; and bottom row - SNAP-25 antibody preincubated with GST-SNAP-25.

SNAP-23 is present in pancreatic acinar cell and SNAP-25 is present in pancreatic islets. Laser scanning confocal microscopy was used to distinguish the distribution of SNAP-25 (in A) and SNAP-23 (in C) in normal (non-diabetic) pancreatic tissue from a rat. These tissue sections were double labeled with guinea-pig anti-insulin antibody to indicate the location of the islets (in B and D).

Subcellular location of SNAP-23 to the basolateral plasma membrane of pancreatic acinar cells by laser confocal microscopy. Pancreatic sections from diabetic rats
with either GST-SNAP-23 (C, F) or GST-SNAP-25 (D) recombinant proteins were performed prior to immunostaining to demonstrate the specificity of the immunostaining. SNAP-25 in a nerve fiber (arrowhead in F) was not blocked by GST-SNAP-23. Double labelling with FITC-phalloidin (B) was performed to indicate the apical portion of the acinar cells (arrows in A and B).
PM  ZGM  Brain  3T3

- Anti-SNAP-23
- Anti-SNAP-23 + GST-SNAP-25
- Anti-SNAP-23 + GST-SNAP-23
- Anti-SNAP-25
- Anti-SNAP-25 + GST-SNAP-23
- Anti-SNAP-25 + GST-SNAP-25
Exercise increases the plasma membrane content of the Na\(^+\)-K\(^+\) pump and its mRNA in rat skeletal muscles

THEODOROS TSASKIRIDIS, PEGGY P. C. WONG, ZHI LIU, CAROL D. RODGERS, MLADEN VRANIC, AND AMIRA KLIP
Division of Cell Biology, The Hospital for Sick Children, Toronto M5G 1X8; Departments of Physiology and Medicine, University of Toronto, Toronto M5S 1A8; and School of Physical and Health Education, University of Toronto, Toronto, Ontario M5S 1A1, Canada

Exercise increases the plasma membrane content of the Na\(^+\)-K\(^+\) pump and its mRNA in rat skeletal muscles. J. Appl. Physiol. 80(2): 699-705, 1996.—Muscle fibers adapt to ionic challenges of exercise by increasing the plasma membrane Na\(^+\)-K\(^+\) pump activity. Chronic exercise training has been shown to increase the total amount of Na\(^+\)-K\(^+\) pumps present in skeletal muscle. However, the mechanism of adaptation of the Na\(^+\)-K\(^+\) pump to an acute bout of exercise has not been determined, and it is not known whether it involves alterations in the content of plasma membrane pump subunits. Here we examine the effect of 1 h of treadmill running (20 m/min, 10% grade) on the subcellular distribution and expression of Na\(^+\)-K\(^+\) pump subunit polypeptides in rat skeletal muscles. Red type I and IIA (red-I/IIa) and white type IIA and IIB (white-IIa/IIB) hindlimb muscles in resting and exercised female Sprague-Dawley rats were removed for subcellular fractionation. By homogenization and gradient centrifugation, crude membranes and purified plasma membranes were isolated and subjected to gel electrophoresis and immunoblotting by using pump subunit-specific antibodies. Furthermore, mRNA was isolated from specific red type I (red-I) and white type IIB (white-IIB) muscles and subjected to Northern blotting by using subunit-specific probes. In both red-I/IIa and white-IIa/IIB muscles, exercise significantly raised the plasma membrane content of the \(\alpha_1\)-subunit of the pump by 64 ± 24 and 55 ± 22%, respectively (\(P < 0.05\)), and elevated the \(\alpha_2\)-polypeptide by 43 ± 22 and 94 ± 39%, respectively (\(P < 0.05\)). No significant effect of exercise could be detected on the amount of these subunits in an internal membrane fraction or in total membranes. In addition, exercise significantly increased the \(\alpha_2\)-subunit mRNA in red-I muscle (by 50 ± 7%; \(P < 0.05\)) and the \(\beta_2\)-subunit mRNA in white-IIB muscles (by 64 ± 19%; \(P < 0.01\)), but the \(\alpha_2\)- and \(\beta_2\)-mRNA levels were unaffected in this time period. We conclude that increased presence of \(\alpha_1\)- and \(\alpha_2\)-polypeptides at the plasma membrane and subsequent elevation of the \(\alpha_2\)- and \(\beta_2\)-subunit mRNAs may be mechanisms by which acute exercise regulates the Na\(^+\)-K\(^+\) pump of skeletal muscle.

sodium-potassium-adenosinetriphosphatase; sodium-potassium pump; red muscles; white muscles; muscle fiber type; contraction; messenger ribonucleic acid

The Na\(^+\)-K\(^+\) adenosinetriphosphatase (ATPase) or Na\(^+\)-K\(^+\) pump [EC 3.6.1.37] is a transmembrane protein complex that functions at the cell surface to main-
whether a single bout of exercise that 1 h of treadmill running (20 m/min, 10% grade) increases the plasma membrane levels of α1- and α2-polypeptides of the pump in both red-I/IIa and white-IIa/IIb muscles. Furthermore, the exercise bout elevates the α1- and β2-mRNA content in red-I and white-IIb muscles, respectively.

METHODS

Materials. Monoclonal antibodies anti-Na+-K+-ATPase-α1 (Mck-1) and anti-Na+-K+-ATPase-α2 (Mch-2) were kind gifts of Dr. K. Sweeney (Boston, MA) (23). Polyclonal anti-Na+-K+-ATPase-β1 and anti-Na+-K+-ATPase-β2, raised against peptides of the extracellular domains of the proteins, were kindly donated by Dr. F. Martín-Vasallo (Tenerife, Spain). The specificity of these antibodies in skeletal muscles was recently examined (20a). The full-length cDNA probes were kind gifts of Dr. J. Lingrell (Cincinnati, OH). Restriction enzymes for the cDNA probes NarI, Stul, Scal, NheI, Ncol and SspI were obtained from Promega (Madison, WI). The materials and chemicals for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were obtained from BioRad (Mississauga, ON, Canada). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Animals and muscle preparation. The week before the day of the exercise bout, 12 female Sprague-Dawley rats weighing 250 ± 25 g were accustomed to treadmill running by one 5-min running period once every 2 days (no running was done the day before the experimental exercise bout). On the day of the experiment, animals were separated randomly into two groups, exercise and resting control (6 animals per group). The exercise group performed 1 h of treadmill running at 20 m/min up a 10% grade. Immediately after exercise or rest, animals were killed by decapitation, and hindlimb muscles were rapidly excised, frozen in liquid N2, and stored at −80°C until use. For membrane fractionation we separated red muscles composed mainly of type I and IIa oxidative and glycolytic (red-I/IIa) fibers (soleus, peroneals, vastus intermedius, and red portions of gastrocnemius, rectus femoris, vastus lateralis, and semimembranosus) and white muscles composed mainly of type I and IIb (white-IIa/IIb) glycolytic fibers (extensor digitorum longus, flexor hallucis longus, tensor fasciae latae, vastus medialis, biceps femoris cranialis, biceps femoris caudalis, and white portions of gastrocnemius, tibialis anterior, rectus femoris, and vastus lateralis) (1). For extraction of total RNA, we used soleus (89% type I) as a representative red (red-I) muscle, and as representative white (white-IIb) muscles, we used a pool of tensor fasciae latae (96% IIb) and white portions of the rectus femoris (99% IIb) and the vastus lateralis (89% IIb) (1).

Membrane fractionation. The day of the subcellular fractionation experiment frozen muscles were removed from the −80°C and were thawed to 0°C. Subcellular membranes were isolated from both red-I/IIa and white-IIa/IIb muscles after a protocol that has been previously established (9, 19) and was recently adjusted for small amounts of tissue (7). Smaller amounts of white-IIa/IIb muscles were obtained from each animal compared with red-I/IIa muscles (1.45 ± 0.18 vs. 2.9 ± 0.13 g for white-IIa/IIb and red-I/IIa, respectively). For this reason, white-IIa/IIb muscles from control or exercised rats were pooled in pairs within each group to allow for the performance of three independent subcellular fractionation and immunoblotting experiments. Membranes isolated by this procedure have been previously characterized through both enzymatic and immunologic approaches (9, 19).

Briefly, a series of differential centrifugation steps yields a crude membrane fraction (CM). Subfractionation of CM on discontinuous sucrose gradients yields plasma membranes (PM; 25% sucrose fraction), which contain plasma membrane markers but not transverse tubules, and an internal membrane fraction (IM; 35% sucrose fraction) depleted of cell surface markers and of sarcoplasmic reticulum, which contains the intracellular pool of insulin-sensitive GLUT-4 glucose transporters and α-subunits of the Na+-K+-ATPase (16, 19).

Immunoblotting. Immunoblotting was performed as previously described (17, 21) and also described briefly here (see Fig. 1). Quantitation of immunoreactive bands was done with a Molecular Dynamics PhosphorImager system (Sunnyvale, CA). To secure accurate estimation of the distribution of pump subunits, the amount of protein transferred to the polyvinylidene difluoride membranes was also quantitated. After immunoblotting, membranes were stained with Protogold (British BioCell International, Cardiff, Wales) and scanned with a Discovery Series (Protein Database) DNA scanner equipped with one-dimension gel-analysis software (ver. 1.3, Huntington Station, NY) as described earlier (15). The immunoblot density values were normalized relative to the detected protein in each lane.

RNA extraction and Northern blotting. Total RNA was extracted from the soleus (red-I) and the pool of three white-IIb muscles (tensor fasciae latae and white parts of rectus femoris and vastus lateralis) by using the single-step RNA isolation with acid guanidinium thiocyanate-phenolchloroform extraction (4). Northern blotting was carried out essentially as described earlier (17). The α1-cDNA probe was generated after restriction of the full-length α1-cDNA with NarI and Stul (to give a probe comprising nucleotides 89-
full-length α-cDNA with ScaI and NheI (to give a probe comprising nucleotides 121–502). The β1-cDNA probe was obtained by incubating the full-length β1-cDNA with restriction enzymes NcoI and SapI (to give a probe comprising nucleotides 459–1,554), and the full-length β2-cDNA (nucleotides 1–1,331) was used. These selected sequences do not cross-react with the alternative isoform transcript (i.e., α1 vs. α2 or β1 vs. β2) (17). Radioactive labeling of cDNA probes was performed by the random primer method (14). After hybridization, nitrocellulose membranes were subjected to three successive 10-min washes with 1× sodium chloride (150 mM)-sodium citrate (15 mM) buffer, pH 7.0, and 0.1% sodium dodecyl sulfate at 65°C before PhosphorImager analysis.

**Statistical analysis.** Statistical analysis was done with paired and unpaired Student's t-test, as indicated.

**RESULTS**

Similar amounts of red-Ia/IIa or white-IIa/Iib muscle tissue from control and exercised rats were isolated and used for subcellular fractionation. The amounts of protein yield in the PM, IM, and CM isolated by the fractionation procedure were also similar between the control and exercise groups (Table 1).

**Effect of exercise on the content and distribution of pump subunits in red-Ia/IIa muscles.** Figure 1A shows representative immunoblots of five independent experiments investigating the effect of exercise on the distribution of α1-, α2- and β1-subunits in PM, IM, and CM isolated from red-Ia/IIa muscles. Exercise significantly increased the content of the α1- (by 64 ± 24%; P < 0.05) and α2-subunits (by 43 ± 22%; P < 0.05) in the PM of red-Ia/IIa muscle when five independent experiments were analyzed (Fig. 1C). Surprisingly, this increase was not associated with a reduction in the content of these subunits in the IM (Fig. 1A) or in the other fractions recovered in the sucrose gradient (results not shown), including the pellet that is rich in sarcoplasmic reticulum markers. Moreover, exercise did not alter the content of α1- and α2-subunits in the CM fraction. Exercise caused a small increase in the PM content of the β1-subunit that, however, did not reach statistical significance when all five experiments were analyzed. Neither the IM levels of β1-subunit nor the total amount of β1-subunit in CM was affected by exercise (Fig. 1A).

**Effect of exercise on the content and distribution of pump subunits in white-IIa/Iib muscle.** Figure 1B shows representative immunoblots from three independent experiments examining the α1-, α2-, and β2-subunit distribution in white-IIa/Iib muscles in the three different experimental conditions. Exercise significantly increased the α1-polypeptide (by 55 ± 22%; P < 0.05) and α2-polypeptide (by 94 ± 39%; P < 0.05) in the PM of white-IIa/Iib muscles in three independent experiments (Fig. 1C). As in red-Ia/IIa muscle, we were unable to detect a reduction in these subunits in the white-IIa/Iib muscle IM (Fig. 1B) or other fractions from the gradient (results not shown). Examination of the CM isolated from white-IIa/Iib muscles also showed no significant alteration in the total amount of α1- and α2-subunits. In the PM, there was a trend of the β2-subunit to increase with exercise, but this did not reach statistical significance. There were no significant alterations in the β2-levels in either the IM or the CM (Fig. 1B).

Whereas immunoblotting does not allow for the calculation of molar equivalents of pump subunits, it does allow one to compare the ratio of β- to α-subunits between different membrane fractions; i.e., one can examine whether this ratio differs between fractions. The ratio of β to α was estimated in control muscles by dividing the optical density reading of the immunoblots, and this ratio was given an arbitrary value of 1.0 for the IM. The ratio of β1 to α1 was found to be 4.1 in the PM compared with 1.0 in the IM of red-Ia/IIa muscles. These values are in arbitrary units and do not indicate molar ratios; however, they do indicate that there are more β1-subunits per α1-subunits in the PM than in the IM. In the same fractions, the ratio of β1 to α2 was found to be 6.7 in the PM compared with 1.0 in the IM (Fig. 1B). In white-IIa/Iib muscles, the ratio of β2 to α1 was 5.7 in the PM compared with 1.0 in the IM, and the ratio of β2 to α2 was 13.3 in the PM compared with 1.0 in the IM. Collectively, these results indicate that there is a relatively greater proportion of β- to α-subunits in the PM than in the IM regardless of β- or α-isofrom.

**Effect of exercise on the expression of pump subunit mRNAs in red-I and white-IIb muscles.** The levels of Na+-K+ pump subunit transcripts were also investigated after the 1-h exercise bout by performing Northern blots of total RNA isolated from soleus muscle (red-I) and tensor fasciae latae or white portions of rectus femoris and vastus lateralis muscles (white-IIb). Figure 2A shows representative Northern blots of the α1-, α2-, and β1-subunit transcripts of control and exercised red-I muscles. In the soleus, the α1-cDNA probe reacted with a single transcript of 3.7 kb. Exercise caused a marked increase in the abundance of the

<table>
<thead>
<tr>
<th>Muscle Mass, g</th>
<th>Protein Yield, µg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Red</td>
<td>5</td>
</tr>
<tr>
<td>White</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. Red-I/Ia muscles of 5 control and 5 exercised animals were examined. Because of small yield of pure white-IIa/Iib muscles from each animal, muscles of 2 control or 2 exercised rats were pooled together to form 3 pairs of white control and exercised muscle groups starting from 6 animals in each case. Average mass of muscles isolated from each group and amount of protein yielded in each subcellular fraction are given. See METHODS for specific red-I/Ia and white-IIa/Iib muscles isolated.
**DISCUSSION**

Electrical stimulation of short duration increases the ouabain-sensitive 
Rb\(^+\) uptake in red and white skeletal muscles (5, 10, 11), indicating increased activity of the
the present study, we began to examine the 

However, the exact mechanism whereby pump 

increase whether there are changes in pump 

is 

Na\textsuperscript{+}-K\textsuperscript{+} pump. This function is likely vital for the 

continued performance of the muscle during exercise. However, the exact mechanism whereby pump activity 

is elevated during acute exercise is largely unknown. In the present study, we began to examine the basis for the 

increase in pump activity during exercise by analyzing whether there are changes in pump subunits at the 

plasma membrane and whether these changes are associated with variations in the mRNA levels in red-I and 

white-IIb skeletal muscles after a single bout of exercise. This became possible because of the recent 

availability of isoform-specific antibodies and cDNA probes to the pump subunits and the development in 

our laboratory of strategies to isolate purified plasma membranes from small amounts of skeletal muscle (7, 

9, 15). These procedures allowed us to analyze muscles composed mainly of type I plus IIa fibers (red) type IIa 

plus IIb fibers (white). We observed that a single bout of exercise can increase the α\textsubscript{1}- and α\textsubscript{2}-protein abundance in 
isolated plasma membranes in both red-I/IIa and 

white-IIa/IIb muscles (Fig. 1), indicating that the plasma membrane Na\textsuperscript{+}-K\textsuperscript{+} pump content can be regulated 

by exercise of short duration. The results suggest that a 

rise in the plasma membrane α\textsubscript{1} and α\textsubscript{2}-subunits can be part of the mechanism of elevation of pump activity 
in response to exercise, particularly because these are the catalytic subunits of the enzyme. These results are 
compatible with earlier findings that ion-pump activity is increased in both red (soleus) and white (extensor 
digitorum longus) rat skeletal muscles after electrically stimulated contraction (12), although quantitative 
differences were noted between the muscles. Our observations are also in agreement with observations of 
increased binding of cardiac glycosides to skeletal muscle of humans undergoing 1 h of bicycle exercise (18). In contrast, there was an increase in the rate of \textsuperscript{3}H]oua-
bain binding to electrically stimulated muscle during the first minutes of stimulation, ascribed to a change in 
pump activity rather than to pump number (11). Because of the differences in the duration and means of
The increase in $\alpha_1$- and $\alpha_2$-subunits at the plasma membrane could be brought about by synthesis of new pump subunits, recruitment of preexisting enzymes from intracellular sites, or stabilization of the plasma membrane-associated complexes in this compartment. Because there was no statistically significant change in pump subunit content in crude (i.e., total) membranes (Fig. 1, A and B), it seems unlikely that new subunit synthesis contributes to the increase in plasma membrane subunit content. On the other hand, our results did not provide information regarding the existence of a donor pool of preexisting pumps because there was no change in subunit content in the intracellular fractions isolated. Yet, it is possible that small membrane pools are not detected by the fractionation procedure. Indeed, there is precedence that there is a pool of intracellular membranes endowed with GLUT-4 glucose transporters that provides transporters to the plasma membrane in response to exercise and is not purified by the isolation procedure used (9).

The observation that $\alpha$-subunits increase in the PM without an apparent concomitant increase in $\beta$-subunits can arise from several hypothetical scenarios. 1) It is possible that $\alpha$-subunits are recruited to the plasma membrane where they associate with free $\beta$-subunits. Indeed, this phenomenon was shown to occur in insect sf-9 cells (8), and there are suggestions for independent intracellular traffic of the $\alpha$- and $\beta$-subunits in response to insulin in mammalian muscle (16). We estimated the relative proportions of the pump subunits in PM and IM. The ratio of $\beta$- to $\alpha$-subunits was severalfold higher in the PM than in the IM regardless of isoform or muscle fiber type. These estimates suggest that the PM compartment may maintain a pool of $\beta$-subunits that could potentially associate with newly recruited $\alpha$-subunits, thereby allowing the formation of functional pump complexes. 2) Our results cannot rule out, however, that the larger proportion of $\beta$-subunits in the PM may obscure detection of a further increase in the PM in response to exercise. Thus we cannot formally discard the possibility that there is translocation of preformed $\alpha$-$\beta$ dimers in response to exercise. 3) Finally, the amount of any protein at the cell surface depends on its rate of cycling through this compartment, determined by the rates of arrival and retrieval. The results obtained are also compatible with an increase in the permanence of $\alpha$-subunits at the plasma membrane in response to exercise, which could be due to decreased rate of retrieval from the plasma membrane. Such a mechanism of regulation of the distribution of pump subunits would also indicate that $a$) the $\alpha$- and $\beta$-subunits of the pump have different rates of arrival to and retrieval from the plasma membrane and $b$) the rate of retrieval of $\alpha$-subunits from this compartment is faster under resting conditions than during exercise.

The regulation of pump subunit content at the plasma membrane by exercise is reminiscent of the stimulation caused by insulin. A 30-min exposure to the hormone $\beta_1$-subunits in mixed (red and white) rat skeletal muscles (16, 21). However, in the case of insulin, the donor pools that supply pump subunits to the plasma membrane were identified (16). Similarly, insulin causes recruitment of the GLUT-4 glucose transporter from an intracellular donor pool, which is recovered with the IM. Thus insulin stimulates the translocation of both glucose transporters and pump subunits from intracellular membrane organelles isolated with the IM, whereas exercise may recruit transporters and pump subunits to the plasma membrane from an intracellular compartment that is not isolated by the present procedure. Future investigations will examine this possibility. Further differences exist between the actions of insulin and exercise on the Na$^+$-K$^+$ pump because insulin recruits to the plasma membrane the $\alpha_2$ and $\beta_1$-subunits (16), whereas exercise increases both $\alpha_1$- and $\alpha_2$- but not $\beta$-subunits (Fig. 1).

In addition to changing the content of $\alpha$-subunits in the PM, the single bout of exercise increased the amount of $\alpha_1$-mRNA in soleus muscle and $\beta_2$-mRNA amount in white-IIb muscles, whereas the $\alpha_2$- and $\beta_1$-transcripts in soleus and the $\alpha_2$-transcripts in white-IIb muscles were unaltered (Fig. 2). The rise in $\alpha_1$-mRNA with exercise in soleus is compatible with earlier studies showing increase in the mRNA levels of this subunit in response to elevated intracellular Na$^+$ in other tissues (6, 22). It is conceivable that elevated intracellular Na$^+$ during exercise may signal the transcription of $\alpha_1$-gene in red-I and the $\beta_2$-gene in white-IIb muscles. The muscle $\beta_2$-isoform increases selectively also in response to thyroid hormone treatment (2). Taken together, our results suggest that biosynthetic mechanisms can be triggered by signals initiated by contraction during a single exercise bout. The lack of concordance between the increase in protein at the plasma membrane and mRNA content suggests that there is regulation of the abundance of subunit transcript during a short exercise bout, which probably does not contribute to change the cellular levels of pumps during this period. This is not without precedence because the abundance of mRNA of the pump (2, 13, 23) and of other gene products such as the GLUT-4 glucose transporter (20) often do not correlate with the levels of the polypeptides they encode. Our results suggest that the pump subunits may be initially regulated posttranscriptionally and that the increase in mRNA may serve as a subsequent mechanism to sustain pump activity through prolonged exercise or during the postexercise period. It is conceivable that elevation in $\alpha_1$- and $\beta_2$-mRNA may in fact lead to increase of the protein content at later stages of exercise, but this was not addressed by the exercise protocol used. Nevertheless, this possibility is compatible with the observation that exercise training augments the number of ouabain binding sites in both rat and human skeletal muscles (Ref. 5 and references therein).

The exact signals involved in the increase of the catalytic subunits of the pump at the plasma membrane in response to exercise need to be investigated.
stimulate the activity of the pump in skeletal muscles (5). These hormones may mediate the effect of exercise on the pump. It has been postulated that the rise in intracellular Ca\(^{2+}\) that takes place during exercise may be a signal involved in the exercise-induced glucose transporter recruitment to the cell surface (24). This possibility should also be considered for the elevation in \(\alpha\)-subunits in this compartment detected in this study.

In conclusion, 1 h of treadmill running increases the plasma membrane content of the catalytic subunits \(\alpha_1\) and \(\alpha_2\) of the Na\(^+\),K\(^+\) pump in both red-I/IIa and white-IIa/IIb skeletal muscles. This is the first evidence that a single exercise bout can regulate the amounts of specific pump subunit isoforms at the plasma membrane. De novo protein synthesis of pump subunits does not seem to be the mechanism for the increase of the \(\alpha_1\) and \(\alpha_2\)-polypeptides in the plasma membrane. Although not directly examined, the results are compatible with either the recruitment of subunits from a yet-untidified intracellular membrane pool or with increased retention of pumps at the cell surface. The exercise bout also elevated the \(\alpha_1\)-mRNA in red-I muscles and \(\beta_2\)-mRNA in white-IIb muscles, perhaps for protein synthesis at a later stage.

We thank Anna Engel, Dimitrios Dimitrakoudis, and Simon Fisher for technical support. We are grateful to Dr. Kathleen Sweenan for the supply of the monoclonal anti-\(\alpha_1\)- and anti-\(\alpha_2\)-antibodies, Dr. P. Martin-Vasallo for the polyclonal anti-\(\beta_2\)- and anti-\(\beta_1\)-antibodies, and Dr. J. Lingrell for the cDNA probes.

This work was supported by grants from the Medical Research Council of Canada to A. Klip (MT-12601) and from the Canadian Diabetes Association to M. Vranic. T. Tsakiris was supported by a University of Toronto Open Studentship and an Ontario Graduate Scholarship.

Address for reprint requests: A. Klip, The Hospital for Sick Children, Toronto, Ontario M5G 1X9, Canada.

Received 22 August 1995; accepted in final form 9 November 1995.

REFERENCES


7. Deems, R. O., R. W. Deacon, T. Ramal, A. Volchuk, A. Klip, and D. A. Young. Insulin action on whole body glucose utiliza-


