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SNARE and Rab Protein Expression in CD8+ T-cells and Natural Killer Cells

by

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Abstract

Rationale: Cytotoxic T-cells (CTL) and natural killer cells (NK) kill their targets through mediators stored in their secretory lysosomes. The latter are composed of secretory granules and lysosomes. My hypothesis is that regulation of docking of CTL and NK cell secretory lysosomes with the plasma membrane involves soluble NSF (N-ethylmaleimide-sensitive factor) attachment proteins receptors (SNAP receptors) (SNAREs) and Rabs. Through the formation of NSF and SNAP complex, v-/R-SNARE (vesicular) and t-/Q-SNARE (target) membrane interactions occur. Rabs matching appropriate v-/R- and t-/Q- SNAREs and are distributed to specific intracellular compartments. **Methods**: Specific primers and RT-PCR were used to determine mRNA expression in human $CD8^+$ clones (CTLs) and the natural killer (NK) cell line, YT-Indy. Western blot and immunocytochemistry were used to confirm the expression of SNAREs. **Results**: CTLs and NK cells express mRNA for all isoforms of SNAREs and Rabs examined. Protein expression was confirmed for VAMP-7, 8, syntaxin 4, 6, and SNAP-23 suggesting that cells containing secretory lysosomes possess machinery found in conventional secretory cells.

Preface

This thesis was written in paper format according to the guidelines of the University of Alberta "Thesis Handbook". Each chapter stands alone as a separate document and is written in the style of the "Immunity".

Acknowledgements

My journey at the University of Alberta has been interesting. I have met a lot of special people who I will never forget. I have learned more than can be put into words, not only in my laboratory training, but also in life. I am truly grateful for the experience and for the friendships that will last a lifetime. Thank you to everyone who has helped me along the way. I am truly indebted.

I would also like to extend a special thanks to my supervisor, Dr. Redwan Moqbel. He has been a great role model and mentor. He has taught me many valuable lessons both in the lab and in life. For this, I am truly thankful.

Table of Contents

Chapter 1

I. Introduction

1.0 *$CD8^+$ Cells (CTL) and Natural Killer cell biology*

2.0 Molecular Mechanisms Underlying the Regulated Release of Granule Contents

Chapter 2

I. Methodology

Chapter 3

Chapter 4

List of Tables

List of Figures

Chapter 1

Chapter 2

Chapter 3

Commonly used abbreviations

APC	Antigen presenting cell
ATP	Adenosine tri-phosphate
β-Hex	β-hexosaminidase
BoNT	Botulinum neurotoxin
bp	base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	copy deoxyribonucleic acid
CLSM	Confocal laser scanning microscopy
CTL	Cytotoxic T-Lymphocyte
FBS	Fetal bovine serum
GrB	Granzyme-B
GTP	Guanine tri-phosphate
IFN-γ	Interferon-γ
Ig	Immunoglobulins
IL	Interleukin
KDa	Kilodalton
LGL	Large granular lymphocytes
M6P	Mannose-6-phosphate
mAb	Monoclonal Antibody
MPR	Mannose-6-phosphate receptor

MHC	Major histocompatibility complex
mRNA	messenger ribonucleic acid
NK	Natural Killer
NSF	N-ethylmaleimide-sensitive factor
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
RIPA	Radioimmunoprecipitation buffer
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SL	Secretory lysosome
SLO	Streptolysin-O toxin
SNAP	soluble NSF attachment proteins
SNAP (23/25)	Synaptosome associated protein
SNARE	Soluble NSF attachment protein receptors
TCR	T-cell receptor
TBS-Tw	TBS + 0.5 %Tween-20
TH	Helper T-Lymphocytes
TI	Toxin-insensitive
VAMP	Vesicle associated membrane

Chapter 1

I. Introduction

1.0 *Cytotoxic T-cell ($CD8^+$ Cells) and Natural Killer cell Biology*

1.1 Lymphocyte Overview

Morphometric and morphological studies have shown that lymphocytes are heterogeneous and can be distinguished from other non-lymphocyte cell populations by haematological staining. Analysis of surface markers and functional tests has identified different lymphocyte subsets. There has been identification of several cytotoxic cell types. Lymphocyte subpopulations include helper T-cells (TH), lymphokine activated killer cells (LAKs), cytotoxic T-cells (CTLs) and natural killer cells (NK). This project will focus on the latter two subpopulations by investigating the complex regulating the docking of granules containing the cytotoxic mediator, granzyme B (GrB) before release from these cells. Most lymphocytes have a 5-10μm diameter, a round nucleus, and a cytoplasm void of granules. Lymphocytes have an extremely high nucleus to cytoplasm ratio, with the latter containing a few organelles such as ribosomes, endoplasmic reticulum, and mitochondria (Ferrarini et al., 1980; Smyth et al., 1993). One subpopulation of lymphocytes (5-10%) is larger in nature and referred to as large granular lymphocytes (LGLs). These lymphocytes have a lower nucleus to cytoplasm ratio, with the cytoplasm containing well-defined organelles and granule populations bound by membranes. In these cells, other small vesicles may surround granule

populations (Carpen et al., 1982; Ferrarini et al., 1980; Huhn et al., 1982; Smit et al., 1983; Zarcone et al., 1987) . The majority of lymphocytes defined as NK or CTL display LGL morphology (Clement et al., 1984; Matutes and Catovsky, 1982; Ortaldo et al., 1981; Polli et al., 1987; Timonen and Saksela, 1980).

Of the four lymphocyte subpopulations mentioned above, T-cells (TH and CTLs) comprise 65-80% of recirculating cells (Bueno and Pestana, 2002). They are located on the inner subcortical region of the lymph nodes and can survive for months or years. CD4 or CD8 (CD = cluster of differentiation) surface proteins separate T-cell subpopulations, which arise after differentiation of progenitor T-cells (Asano et al., 1996; Chen et al., 2001; Suto et al., 2001; Thornton and Shevach, 1998). Morphologically, both T-cell populations (TH/$CD4^+$ and CTL/$CD8^+$) express the CD3 surface glycoprotein, which is associated with the T-cell receptor (TCR) and transmits outside-in signals following TCR ligation (Levinson, 1998). Functionally, T-cells can be generally categorized as either regulatory or effector cells separated on the basis of their surface proteins expression (Asano et al., 1996; Chen et al., 2001; Hamid et al., 2003; Suto et al., 2001; Thornton and Shevach, 1998). In addition, TH cells activate cell populations including, $CD8^+$ cells and other $CD4^+$ cells by producing IL-2, which sustains the differentiation and proliferation of $CD8^+$ cells (Morgan et al., 1976) (Figure 1.1).

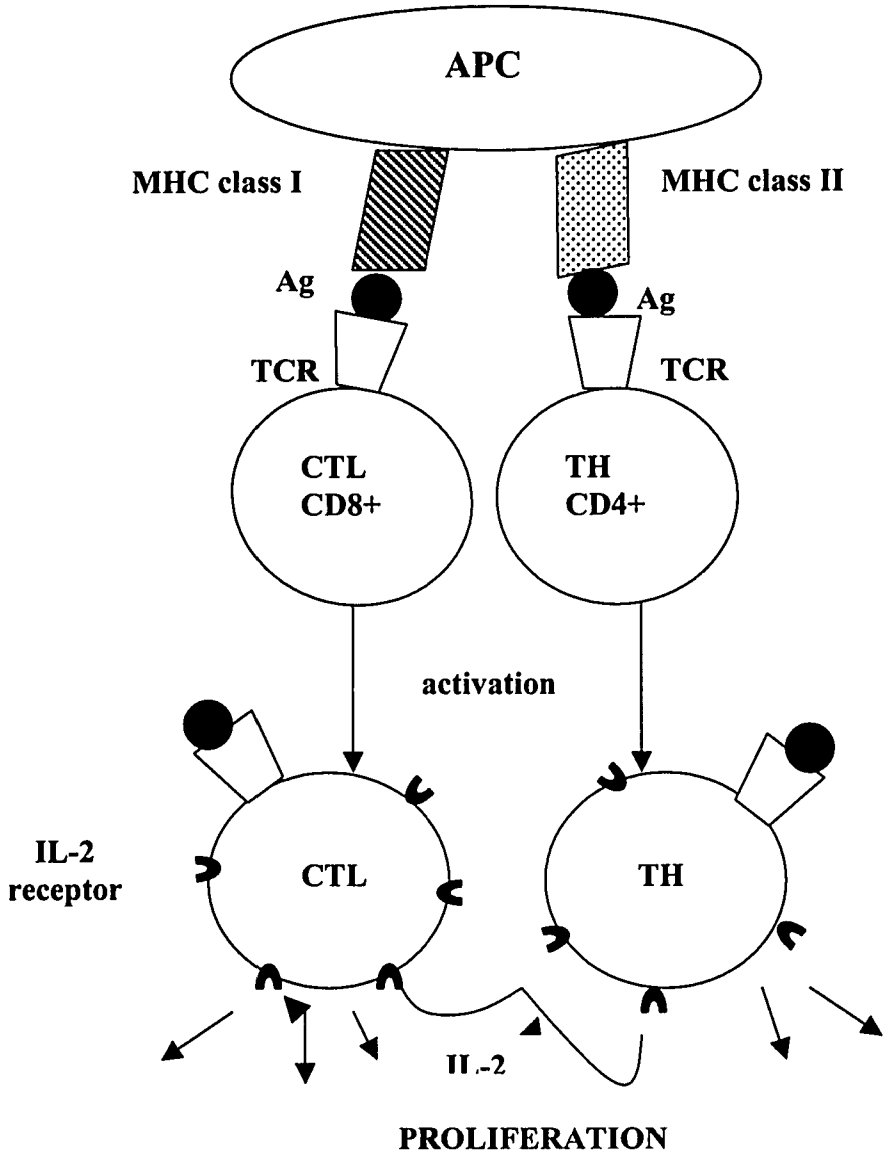

Figure 1.1 T-lymphocyte recognition of antigen.

$CD4^+$ cells (helper T-lymphocytes, TH) recognize foreign antigens in the context of major histocompatibility complex (MHC) class II, while $CD8^+$ (CTL) cells recognize antigen in the context of MHC class I. Antigen presenting cells (APC) breakdown phagocytosed proteins and present epitopes to T-cells via the T-cell receptor (TCR). CTLs cells are functionally cytotoxic and can repeatedly kill cells expressing processed antigen. TH activates CTLs by producing IL-2. IL-2 production by the TH cells sustains the differentiation and proliferation of CTLs (Adapted from: Vollenweider and Groscurth, 1991).

CTLs are effector cells that can ultimately wield their cytotoxic effect by acting against altered cells target cells including, virus-infected cells, tumour cells and cell-mediated allograft rejections (Vollenweider and Groscurth, 1991). Foreign proteins are cleaved into small peptides upon entry into a cell. $CD4^+$ cells recognize foreign antigens (Ag) in the context of MHC class II, while $CD8^+$ cells recognize antigen in the context of MHC class I. Antigen presenting cells (APC) breakdown and denature ingested proteins and peptide-MHC complexes to T-cells. APCs express B7 (CD80), which binds to CD28 on TH cells. Costimulatory molecules must interact for activation to occur and induce IL-2 synthesis by T-cell (Levinson, 1998). Surface TCR recognize the epitopes through polypeptide chain interactions. A mature T-cell can only recognize a limited range of epitopes due to receptor gene rearrangements. MHC molecules can associate with self and non-self proteins. Thus, T-cells must adopt strategies to eliminate the recognition of self- epitopes (Corrigan, 1997). Autoreactive T-cells are functionally inactivated or die by clonal anergy or clonal deletion (Blackman et al., 1990; Kappler et al., 1988; Kisielow et al., 1988; MacDonald et al., 1988; McDuffie et al., 1987).

This project is primarily concerned with CTL and NK cells, which are lymphatic lineage-derived cytotoxic effector cells. Although these cells may show morphological similarities, they differ functionally in their target cell spectrum, surface molecule expression, and mechanisms of target cell recognition. Despite these functional differences, CTLs and NK cells share the common cytotoxic mediator's GrB and perforin, stored within unique compartments termed the secretory lysosomes. In addition, they have the common feature that both must bind to the target cell in order to kill it (Vollenweider and Groscurth, 1991). Release of GrB into the target cell leads to

cell death mediated by the cleavage and activation of caspases. Another pathway also exists resulting in mitochondrial collapse and the release of cytochrome c, activating pro-apoptotic family members (Barry and Bleackley, 2002).

1.2 CTLs ($CD8^+$)

About 35% of peripheral T-cells are $CD8^+$. This T-cell subpopulation is found primarily in lymphoid tissue and bone marrow (Levinson, 1998). The definition of this distinct T-lymphocyte subset is the presence of the TCR, used for Ag recognition along with CD8. Ag presented with MHC class I is recognized by CTLs via the TCR (Vollenweider and Groscurth, 1991). CTLs are efficient killers with a high degree of specificity to target cells, killing in a sequential manner (Fleischer, 1986; Rothstein et al., 1978; Sanderson, 1976). There are two main mechanisms of CTL killing.

The first pathway of CTL mediated killing leads to apoptosis (programmed cell death) of target cells through the release of soluble effector molecules, perforin and GrB from granule populations. By releasing these two cytolytic proteins, this results in plasma membrane damage and nuclear disintegration of target cells. In this pathway, CTLs recognize class I MHC, complexed with Ag on foreign cells (Levinson, 1998). Naïve $CD8^+$ T-cells are able to acquire specialized compartments and synthesize cytotoxic proteins only after: (a) recognition of the antigen bound to MHC class I by the TCR and, (b) interactions between co-stimulatory molecules present on the surface of the APC and T-cell (Griffiths, 1997) (Figure 1.2).

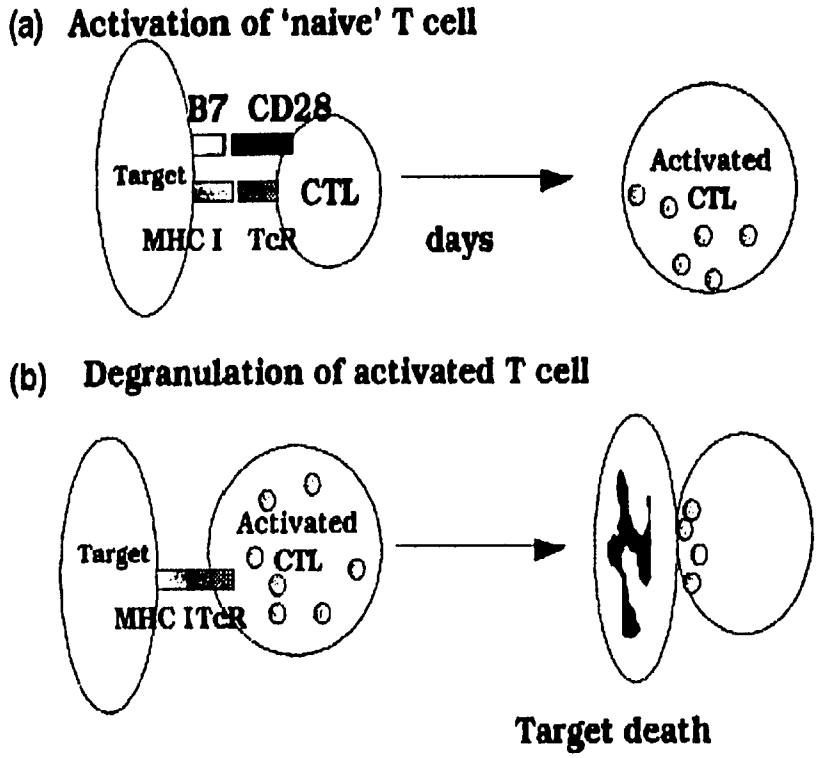

Figure 1.2 $CD8^+$ cell activation.

Activation of a naïve T-cell requires several things to occur. (a) The first is recognition of antigen in the context of class I MHC (on the target or APC) by the T-cell receptor (TCR). The second step is that costimulatory molecules B7 on the APC and CD28 on the CTL must interact. At this time, the CTL can begin to synthesize cytotoxic mediators leading to the activation of the CTL. (b) Upon a second encounter with the antigen a mature cell then can cross link MHC and the TCR with out the costimulatory molecules resulting in degranulation, protein synthesis and turnover of new granules to replace those that are released and target cell death (Griffiths, 1997).

Secretion of IL-2 by TH cells activates the release of degradative enzymes (perforin and granzymes) from CTLs. These enzymes are stored in a unique organelle generally found in cells of the haematopoietic lineage (Blott and Griffiths, 2002). A CTL can continue to kill other cells infected with the same antigen.

The second mechanism of CTL mediated killing occurs through Fas (CD95)-Fas ligand (FasL) (Griffiths, 1997). Calcium, perforin, and granzyme are not required for this pathway (Helgason et al., 1992; Ostergaard et al., 1987; Rouvier et al., 1993). Fas expressed on the target cell surface will bind FasL after a specific epitope is recognized by the TCR on the CTL. Similar to the granule exocytosis pathway, this stimulation of surface receptors leads to activation of pathways leading to target cell death (Barry and Bleackley, 2002). CTL killing is predominantly through granule exocytosis, however, if the latter pathway is compromised, then the Fas pathway is initiated (Barry and Bleackley, 2002; Smyth et al., 2005). This project is primarily concerned with the first pathway, which involves target cell apoptosis through regulated exocytosis of CTL and NK cell granules contents.

1.3 Natural Killer Cells

Natural killer cells (NK) are LGLs that are non-antigen specific. They are capable of responding to tumour or virus-infected cells before the initiation of the adaptive immune response (Smyth et al., 2005; Vollenweider and Groscurth, 1991). LGL morphology was observed in the majority of NK cells with Giesma staining (Vollenweider and Groscurth, 1991). NK cells do not express the TCR, but have their

own characteristic surface molecules, including CD16 and CD56, which can help classify NK cells into different subpopulations (Vollenweider and Groscurth, 1991). CD16 (FcγRIII), may mediate antibody-dependent cytotoxicity, while CD56 helps to identify a unique peripheral blood lymphocyte population (Campbell et al., 2001). Functionality tests have demonstrated cytotoxic activity in NK cells expressing combinations of surface markers CD16, CD56, CD57 and CD11b (Abo and Balch, 1981; Breard et al., 1981; Kang et al., 1985; Manara et al., 1986; Ramsdell et al., 1988). The wide variety of surface receptors on NK cells provides positive and negative signals to the cell, regulating its activation (Smyth et al., 2005). In addition, NK cells have the ability to kill target cells that do not express MHC on their surface (Vollenweider and Groscurth, 1991). Activation of NK cells occurs when NK-specific ligands and reduced levels of MHC class I are expressed on a target cell (Smyth et al., 2005; Smyth et al., 2002). Granule exocytosis is needed to induce target cell death in NK cells (de Saint Basile and Fischer, 2001) (Figure 1.3). NK cells are similar to CTLs in that they possess a single organelle, termed the secretory lysosome (SL), which provides both degradative and secretory functions to the cell (Smyth et al., 2005). Perforin and GrB released from the SL mediate target cell death.

Cytotoxic granules in NK cells are preformed during cell maturation rather than synthesized de novo upon target cell recognition and are released upon activation (Russell and Ley, 2002; Smyth et al., 2005). Biron et al. 1999, have shown numerous cytokines to be involved in mediating cytotoxicity and regulating cell proliferation such as IL-12, a regulatory cytokine that helps regulate and initiate the immune response (Biron et al., 1999). Cytotoxicity by NK cells occurs quickly and effectively, inducing

the adaptive immune response thereby limiting the spread of the infected cell (Smyth et al., 2005) (Figure 1.4).

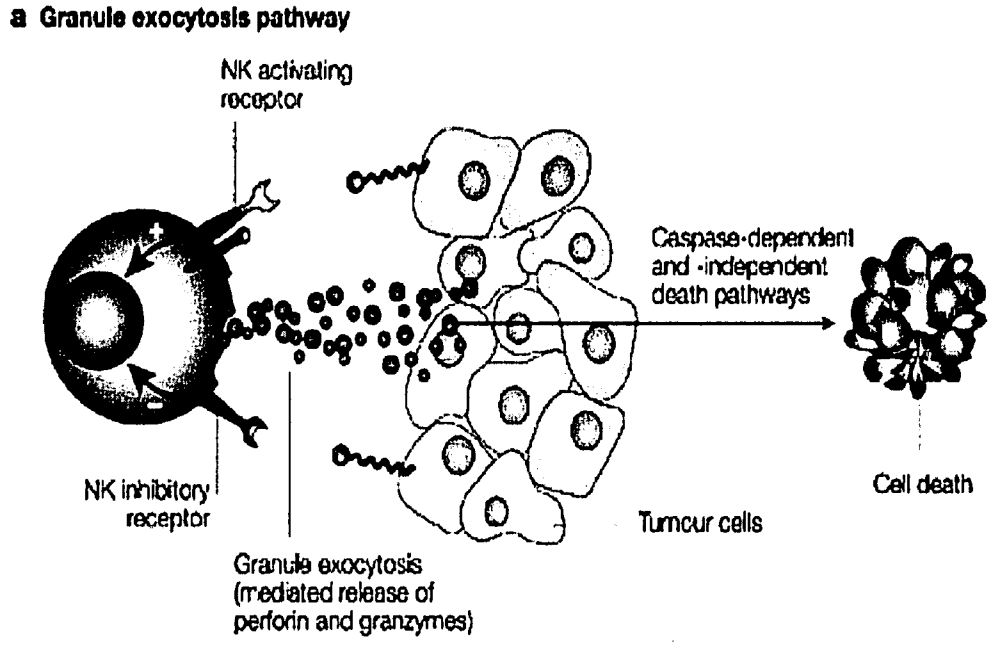

Figure 1.3 NK cell elimination of targets.

Granule exocytosis pathway using perforin and GrB. Cytotoxic granules are oriented towards the target cell. Perforin disturbs the target cells membrane allowing entry of GrB into the target cell. This pathway is calcium dependent (Smyth et al., 2002).

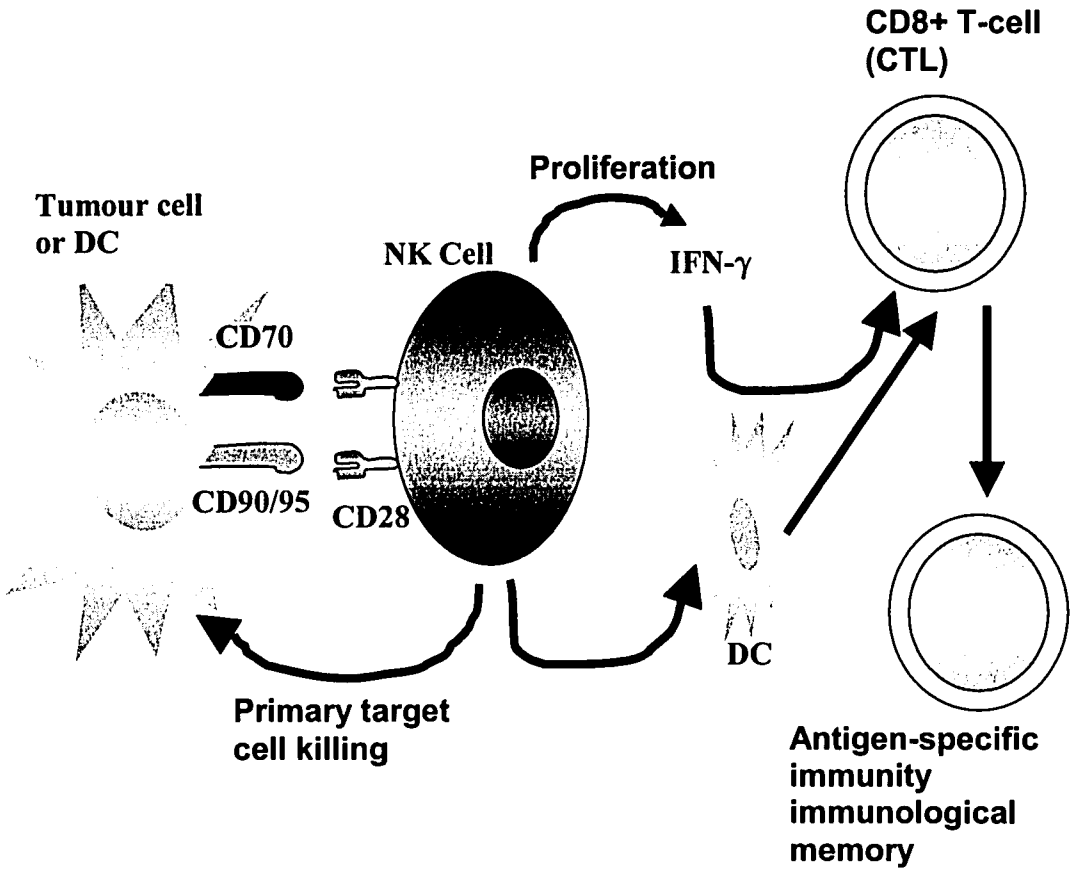

Figure 1.4 Linking the innate and acquired immune system by regulation of NK cells through co-stimulatory molecules.

A model for NK cell activation. Surface molecules on tumour cells or dendritic cells (DC) activate NK cells. These molecules interact with molecules on NK cells. NK cells are activated and release cytokines or kill the infected cell. DC-NK cell interaction can result in DC maturation, which allows the development of $CD8^+$ responses (Adapted from: Smyth et al., 2002).

1.4 Granzyme-B (GrB)

Granzymes are located in the cytoplasm of CTLs and NK cells, with 11 identified so far. GrB is the granzyme of interest of this project. The homology of other granzymes with GrB is 38-67%. Granzymes contain a catalytic triad of serine proteases, His-57, Asp-102, and Ser-195 (Kam et al., 2000) (Figure 1.5). GrB is among the first granzyme to be expressed during cellular maturation (Prendergast et al., 1992) (Figure 1.6). The type of substrate cleaved and the site of cleavage can be used for the identification of a granzyme (Kam et al., 2000). The enzymatic activity that GrB possesses is aspartase (Asp-ase), which cleaves after aspartate (Asp) (Fruth et al., 1987; Hudig et al., 1987; Masson and Tschopp, 1987; Odake et al., 1991; Poe et al., 1988; Poe et al., 1991; Smyth et al., 1993; Woodard et al., 1998).

CTL and NK cells both possess GrB and can mediate killing through the granule exocytosis pathway. During cytotoxic lymphocyte killing, a secretory process is triggered upon target cell recognition (Henkart et al., 1995; Nakajima et al., 1995; Smyth and Trapani, 1995; Trapani et al., 1996; Tschopp and Jongeneel, 1988). Cytotoxic molecules are released when cytoplasmic granules fuse with the plasma membrane of the effector cell (Henkart, 1985).

The other cytotoxic molecule stored in CTL and NK SLs is perforin. Perforin is a first synthesized as a 70kDa immature protein, and yields a 60kDa active form when cleaved. It becomes activated and cleaved only after reaching the acidic compartment of the endosome (Henkart et al., 1984; Uellner et al., 1997). Neutralization of secretory products is maintained by the acidic pH, which is optimal for hydrolases, while neutral conditions favour perforin and granzyme function, which is achieved during exocytosis

(Masson et al., 1990). Other granule mediators have been suggested to play a role in cytotoxic lymphocyte mediated lysis (Hayes et al., 1989; Martz, 1977; Russell, 1983). It was generally accepted that membrane damage is induced by perforin allowing granzyme entry into the cell where it can induce target cell death pathways (Kam et al., 2000) (Figure 1.7). In this case, perforin forms pores upon contact with the target cell membrane such that GrB can be taken up into the target cell (Podack, 1992; Willoughby et al., 2002; Yagita et al., 1992). However, it has been shown that in the absence of perforin, various granzymes can still enter target cells (Edwards et al., 1999; Froelich et al., 1996; Shi et al., 1997). However, these granzymes failed to induce apoptosis or cytotoxicity in the absence of perforin (Pinkoski et al., 1998). Subsequent treatment with perforin resulted in apoptotic-like features, that implicate perforin as a release molecule for stored granzyme (Froelich et al., 1996; Jans et al., 1998; Pinkoski et al., 1998; Shi et al., 1997). This suggests that perforin acts to release internalized GrB are therefore jointly required to induce apoptosis of target cells. Recently, a receptor for GrB has been identified suggesting that the uptake of this protease can occur through receptor mediated endocytosis and perforin acts to release internalized granzymes (Motyka et al., 2000).

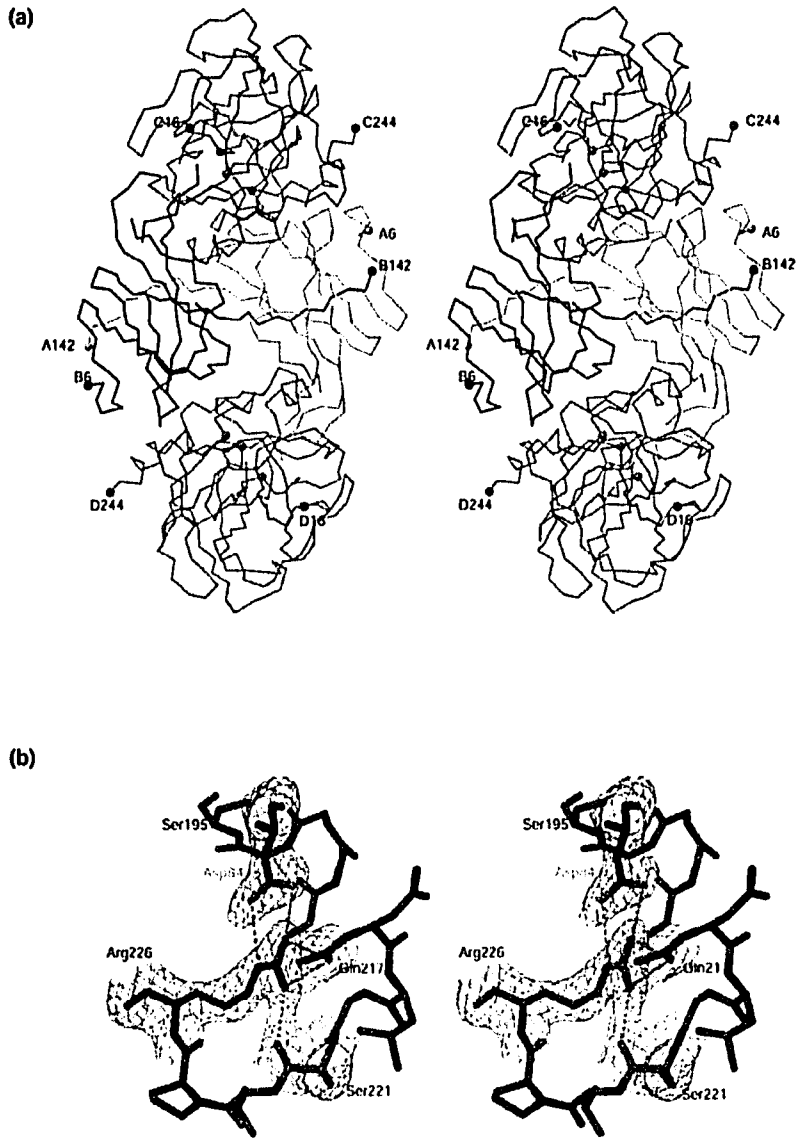

Figure 1.5 GrB crystal structure.

(a) GrB (green) complexed with an inhibitor (blue). GrB catalytic sites (orange). (b) A 2.2 Å electron density map shows the GrB catalytic sites. Arg226 (green) is held by three hydrogen bonds (from Gln217 and Ser221). Aspartic acid is blue (Trapani, 2001).

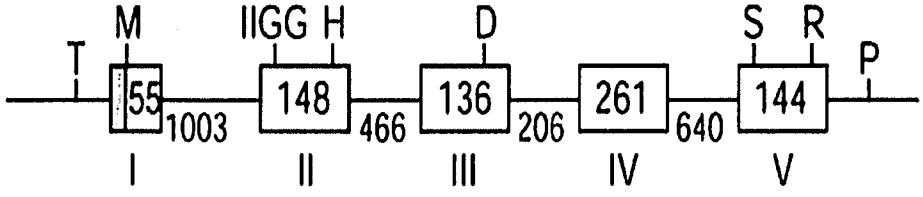

Figure 1.6 The human GrB gene.

The gene is approximately 3.2kb in length. The TATA box (T) is present about 30bp from the start of the transcription site. A total of 5 exons are present encoding the leader sequence (M), the mature protein start site (IIGG), histidine (H) the first of the catalytic triad, aspartic acid (D), and the last catalytic residue serine (S). The polyadenylation signal is P. Exon and intron lengths are shown in base pairs. The 5' untranslated sequence is shaded. Arg226 side chain (R) (Trapani, 2001).

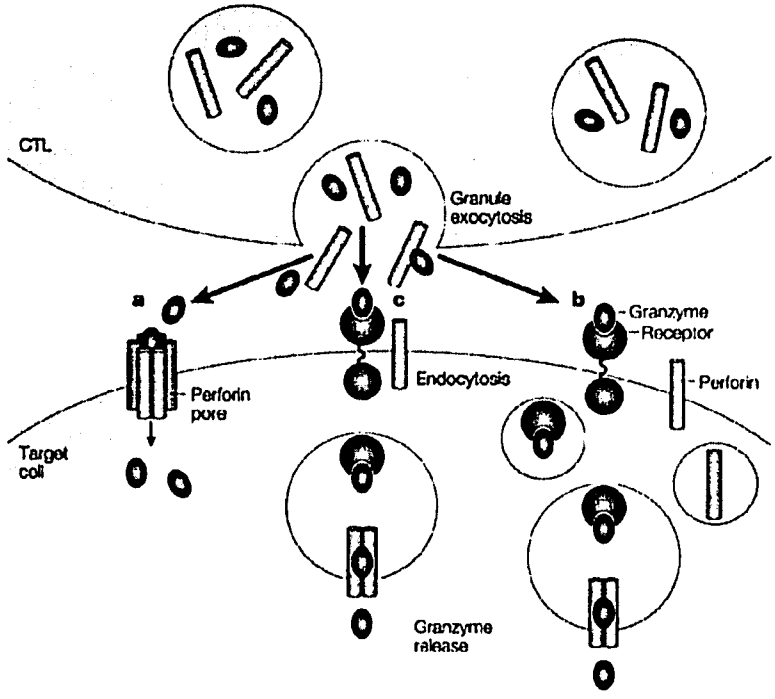

Figure 1.7 Granzyme B entry pathways.

CTL granules are exocytosed upon interaction with the target cell. There are three pathways of entry (a) GrB pass through the target-cell membrane after perforin forms a pore in the plasma membrane (b) receptor-mediated endocytosis model where GrB is internalized by receptor-mediated endocytosis and perforin releases granzyme present in endosomes located in the target cell cytoplasm. (c) GrB can enter the cell stimulated by perforin mediated plasma membrane damage. Upon target cell membrane repair, perforin and GrB get internalized (Barry and Bleackley, 2002).

1.5 The Secretory Lysosome (SL) (Lytic Granules)

The SL is a unique lysosomal organelle found in haematopoietic cells including CTLs and NK cells. SL performs dual functions. It has a dense core that resembles other secretory granules and stores secretory proteins. As well, it has multivesicular cortices which resemble lysosomes functioning to degrade proteins, transports undigested material to cell membrane for removal, digests internalized particles (Blott and Griffiths, 2002; Burkhardt et al., 1990; Peters et al., 1991; Peters et al., 1989). SLs are similar to conventional lysosomes in their acidic pH and their contents of both membrane plus soluble hydrolases, presumably for lysosomal degradation (Burkhardt et al., 1990). In addition, SL and lysosomes are the end-point of the endocytic pathway (Stinchcombe et al., 2000). The main differences between conventional lysosomes and SL are that the latter are much larger in size and have a more complex structure than the traditional lysosome. In addition, early in the endocytic pathway, SLs are initially positive for the mannose-6-phosphate receptor, used for sorting secretory granules. Standard lysosomes lack this receptor. Finally, SLs are able to undergo regulated secretion and fusion with the plasma membrane, whereas conventional lysosomes are not generally associated with plasma membrane fusion (Blott and Griffiths, 2002; Groscurth et al., 1987).

Morphologically, both SL and conventional lysosomes appear similar. However, SL structures are quite diverse ranging from dense cored to multilaminar, while lysosomes usually have multi-vesicular structure. In addition to structural differences of SL and lysosome, these two populations also differ in internal machinery. In addition to standard resident proteins found in both lysosomes and SL, the latter also possess secretory components including GrB (in CTLs and NK cells) (Figure 1.8). The main

difference between lysosomes and SL is that the latter are able to secrete their granule component (Blott and Griffiths, 2002; Bright et al., 1997; Mullins and Bonifacino, 2001; Stinchcombe et al., 2000). It has been suggested that since all lysosomes in CTLs have secretory proteins, CTLs and NK cells only have SL. However, it is interesting to note that other cell types, including melanosomes and platelets, also contain traditional lysosomes and SL suggesting that it is possible to harbour two distinct populations of lysosomes within the same cell (Raposo et al., 2001).

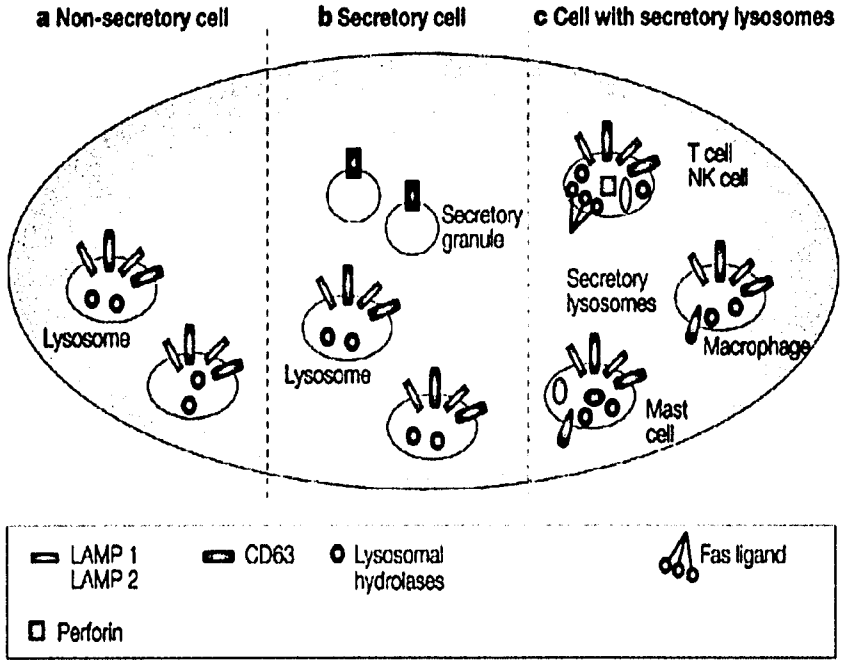

Figure 1.8 Comparison of non-secretory, secretory and secretory lysosome containing cells.

(a) Non-secretory cells contain lysosomes with traditional lysosomal proteins ($LAMP^+$, $CD63^+$) and hydrolases. (b) secretory cells have two separate granule components, secretory granules in addition to traditional lysosome (c) cells with secretory lysosomes have one organelle type, which is a combination of lysosome and secretory granule and contains both lysosomal proteins ($LAMP^+$ and $CD63^+$) and lysosomal hydrolases and secretory proteins (GrB/perforin) (Blott and Griffiths, 2002; Stinchcombe and Griffiths, 2001).

1.6 Sorting Proteins to Secretory Lysosomes (Lytic Granules)

SLs have distinct sorting and secretion pathways from traditional secretory granules. Endocytic or biosynthetic routes can direct proteins into secretory lysosomes (Stinchcombe and Griffiths, 1999). Two distinct pathways mediate sorting of soluble proteins to lytic granules (Figure 1.9). The main pathway occurs through the mannose-6-P receptor (MPR). This receptor is also responsible for targeting lysosomal proteins (hydrolases) to the lysosomes (Kornfeld, 1990). Similarly, granzymes are modified by mannose-6-phospahte (M6P) while passing through the *cis*-Golgi. Granzyme N-linked glycans receive the modification, which occurs through a phosphotransferase (Griffiths, 1997). The phosphotransferase is specific for lysosomal hydrolases and granzymes. As the granzymes and lysosomal proteins pass through the Golgi, they receive the M6P modification. As these protein enter the *trans*-Golgi, network (TGN) M6P modified proteins are recognized by the MPR. Upon entry of the proteins into the acid compartment of the pre-lysosome, the M6P modified proteins release from the MPR. The MPR then cycles back into the TGN (Griffiths, 1997).

Griffiths and Isaaz, (1993) performed studies on CTLs from patients having a genetic mutation known as I-cell disease. This disease, also known as mucolipidosis, is a result of missing or defective phosphotransferase. In these patients, lysosomal hydrolases do not receive the M6P modifications and therefore the MPR does not recognize the hydrolases. Consequently, constitutive secretion of lysosomal proteins occurs since these proteins can not be sorted to the lysosomes even though the MPR is functional (Griffiths and Isaaz, 1993). Moreover, constitutive secretion of GrB was also observed in CTLs derived from I-cell patients. This demonstrated that similar to

lysosomal proteins, GrB is also sorted to the lytic granules via the MPR pathway (Griffiths and Isaaz, 1993). An MPR-independent pathway was also purposed stemming from observations of CTLs from I-cell patients. The bulk of the GrB and lysosomal hydrolases were constitutively secreted, however, low levels of regulated secretion were also observed from granules indicating an alternative MPR-independent pathway of sorting. Although two pathways do exist, the MPR-dependent pathway is the dominant pathway.

It is unclear as to how perforin is sorted to lytic granules, however, it is thought that perforin is also sorts thought the MPR-independent pathway (Blott and Griffiths, 2002). Perforin N-linked glycans are not modified by M6P and, therefore, exclusively use an M6PR-independent pathway to reach the lysosomal compartment (Griffiths, 1997).

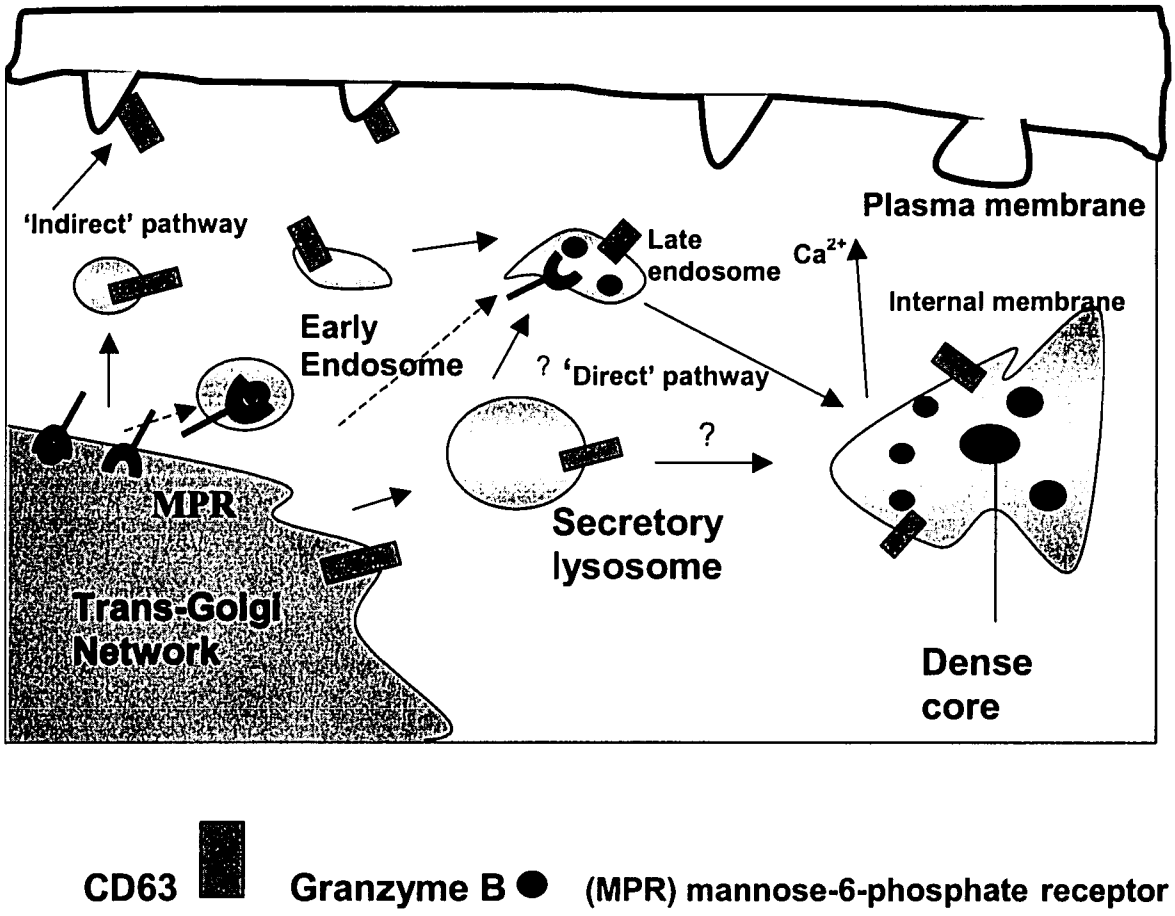

Figure 1.9 Sorting pathways to the secretory lysosomes.

Secretory lysosomes (SL) proteins (GrB) are modified by the mannose-6-phosphate (M6P) network and recognized by the M6P-receptor (MPR) at the *trans*-Golgi network (TGN). Modified proteins are transported to the late endosome (dashed arrow), dissociate from their receptor, and delivered to the SL. Membrane proteins reach the secretory lysosomes by two main routes: direct or indirect. In the direct pathway, it is unknown if the route is through the TGN to the SL or whether it involves other intermediate steps through the endosome. A protein that uses the indirect route travels to the plasma membrane where it is internalized and reaches the SL (Adapted from: Blott and Griffiths, 2002; Smyth et al., 2002).

1.7 The Secretory Processes

Regulated secretion is necessary in CTL and NK cells because uncontrolled release of cytotoxic granule contents can induce apoptosis of bystandard cells as well as cause self destruction (Bossi and Griffiths, 1999; Thompson and Allison, 1997). SL release their contents within minutes of encountering the target cells and destroy it (Griffiths, 1997). Once a target has been recognized a signal is received through the TCR in CTLs for example, which initiates the secretory process (Blott and Griffiths, 2002) (Figure 1.10). This signal also eventually leads to degranulation and protein synthesis, therefore, new granules immediately replace released ones (Griffiths, 1997). Stimulation of the TCR leads to mobilization of Ca^{2+} within the cell. Ca^{2+} binds to sensors present on the granules (Lyubchenko et al., 2001; Yoshihara and Montana, 2004) signaling the granules to begin mobilization to the site of stimulation (Lyubchenko et al., 2001). CTLs rapidly polarize their microtubule organizing centre (MTOC), Golgi and granules towards the target cell (Burkhardt et al., 1993; Geiger et al., 1982; Kupfer and Singer, 1989; Yannelli et al., 1986). Secretory lysosomes move along the microtubules toward the plasma membrane. When the SL get close enough to the plasma membrane they detach from the microtubules, dock, and fuse with the plasma membrane (Griffiths, 1997). Perforin delivery occurs after the effector cell cytoplasm undergoes rearrangement (Barry and Bleackley, 2002). Membrane contact between the CTL cell and the target cell is followed by release of granules into the extracellular space between the target and the CTL (Griffiths, 1997). Effectiveness of the killing is determined by efficiency of delivery to target and the potency by which they deliver (Griffiths, 1997).

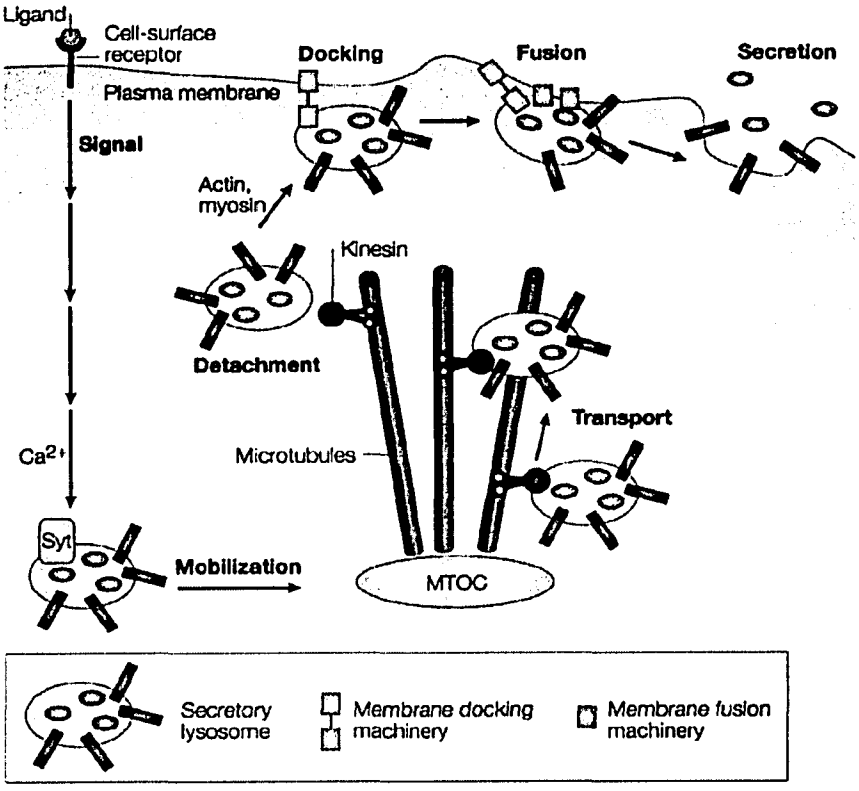

Figure 1.10 CTL secretory process.

Polarization of the microtubule organizing centre (MTOC) and Golgi leads to polarization of granule towards the contact site. This is followed by the release of granules into target cell. Perforin delivery occurs after the effector cell cytoplasm undergoes rearrangement leading to granule exocytosis (Blott and Griffiths, 2002; Stinchcombe and Griffiths, 2001).

2.0 Molecular Mechanisms Underlying the Regulated Release of Granule Contents

2.1 Exocytosis: An Overview

Exocytosis is the final step of the secretory pathway in which the plasma membrane fuses with granules derived from the trans-Golgi (Moqbel and Lacy, 1999). For exocytosis to occur, vesicles must travel to the site of plasma membrane fusion (Zerial and McBride, 2001). Four distinct steps are involved in exocytosis of granules including (Figure 1.11). (1) the formation of an intermediate vesicle, (2) movement of the vesicle towards the target, (3) tethering/docking to the target membrane, which begins the process of exocytosis. This includes contact of the outer leaflet of the lipid bilayer membrane surrounding the granule matrix with inner leaflet of the plasma membrane (Moqbel and Lacy, 1999) followed by (4) membrane fusion (Whyte and Munro, 2002; Zerial and McBride, 2001). Exocytosis in cells containing large secretory organelles involves cycles of rapid opening and closing of fusion pore known as flickering (Jahn and Sudhof, 1999). There is debate over the supposed channel-like formation of the fusion pore; nonetheless, the fusion pore allows lipid transfer between the plasma membrane and the secretory vesicle undergoing fusion. After joining of the plasma and the granule membrane, the fusion pore enlarges and the interior of the granule membrane is exposed to the exterior and its contents expelled (degranulation) (Moqbel and Lacy, 1999).

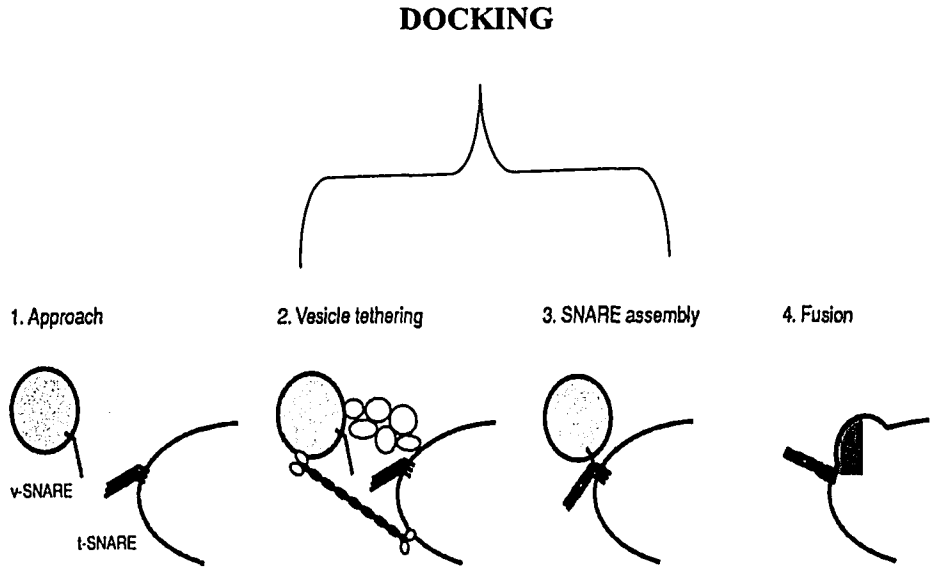

Figure 1.11 Steps in vesicle secretion.

(1) Vesicle approaches target. (2) Vesicle tethering by protein complexes. (3) v-SNARE (on vesicle) and t-SNARE (on target) interact bringing the vesicle membrane and the target cell membrane together. (4) Membrane fusion. Steps 2 and 3 are also known as "docking" (Adapted from: Whyte and Munro, 2002).

A cell can undergo constitutive or regulated exocytosis. In both types of exocytosis, either the endosomal compartment or the TGN is the origin of the vesicles undergoing exocytosis (Fischer von Mollard et al., 1994). Despite origin similarity, regulated and constitutive exocytosis are distinct. The main difference is that in constitutive exocytosis calcium levels can be reduced and the process continues unimpaired, while in regulated exocytosis a rise in calcium levels is necessary (Lledo et al., 1994). This project is concerned with the final steps of regulated exocytosis of SL from CTL and NK cells, namely the docking and fusion machinery.

Control of membrane fusion events in regulated exocytosis occurs through recognition and membrane targeting of specialized proteins (Fischer von Mollard et al., 1994). Regulatory proteins involved in triggering exocytosis are important to identify to ensure regulated release of granule contents (Moqbel and Lacy, 1999). Over the last decade, a number of proteins have been identified in exocytotic membrane fusion, including the SNARE complex (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors) as well as Rab proteins (Jahn and Sudhof, 1999), which share similar molecular mechanism in all eukaryotes (Fischer von Mollard et al., 1994). Interestingly, constitutive fusion has not yet identified docking intermediates, most likely due to the quick disassembly of the SNARE complex in an unfavourable energy state. Since regulated vesicle fusion requires the influx of Ca^{2+} to trigger vesicle fusion, docked vesicles are prevented from immediate fusion as fusion (Goda, 1997).

2.2 Neuronal SNAREs

Molecules involved in vesicular transport and exocytosis events are highly conserved from yeast to man (Rothman, 1994; Scheller, 1995; Sudhof, 1995). SNAREs are integral membrane proteins (Rothman and Wieland, 1996). They are a group of membrane-bound proteins originally categorized into two groups based on their localization. V-SNAREs are vesicle-specific receptors and t-SNAREs are target membrane-specific receptors (Rothman and Warren, 1994; Sollner et al., 1993). Through the formation of NSF and the SNAP complex, v-SNARE and t-SNARE interactions can occur and contact is established between vesicles and the target membrane (Jahn and Sudhof, 1999). These finding lead Rothman and colleagues to propose the SNARE hypothesis to explain events involved in docking and fusion of vesicles (Sollner et al., 1993). Generally, it is agreed that there are four components to hypothesis. (i) NSF (N-ethylmaleimide-sensitive fusion protein), an ATPase with two ATP binding sites), which are required for membrane fusion (ii) SNAPs (soluble NSF attachment proteins), which act as adaptors for NSF and are involved in regulation of exocytosis, (iii) t-SNAREs, which target membrane proteins consisting of two or three polypeptides, and (iv) v-SNAREs, which are vesicle membrane proteins consisting of a tail-anchored SNARE with a single SNARE motif (Fukuda et al., 2000; Goda, 1997) (Figure 1.12). The SNARE complex is energized by ATP hydrolysis (Goda, 1997). V- and t-SNARE come together for vesicle docking and act as receptors for SNAPs and the fusion protein NSF (Goda, 1997). The *trans*-SNARE complex (SNAREpin) is formed through the interaction of the v- and t-SNARE (Weber et al., 1998). A SNARE complex includes three membrane proteins bound together, namely two t-SNAREs and one v-SNARE.

Formation of this complex allows initiation of the fusion process by serving as a docking site for vesicles (Sogaard et al., 1994). Neuronal SNAREs acting at the synapse best illustrate vesicle targeting and fusion specificity (Jahn and Sudhof, 1999).

In order for NSF to function, it must bind to its adaptor protein, SNAP, to be then transported to the site of action. Three isoforms of SNAP exist. All cell types express α-SNAP and γ-SNAP, however β-SNAP is brain specific (Rothman, 1994). Sollner et al. 1994, identified synaptobrevin/VAMP-2 (a v-SNARE), and two plasma membrane proteins, syntaxin, and synaptosome associated protein, SNAP-25, (t-SNARES) to be membrane receptors for NSF-SNAP (Sollner et al., 1993). Conformation of the SNAREs involvement in vesicle docking is supported by the fact that these three SNAREs are targets for cleavage by Clostridial toxins. Tetanus toxins cause rigid paralysis, while Botulinum toxins cause flaccid paralysis, depending on the site of SNARE cleavage (Montecucco and Schiavo, 1995). Cleavage of SNAREs by these toxins provided critical evidence of the importance of SNAREs in vesicle fusion (Goda, 1997) (Figure 1.13).

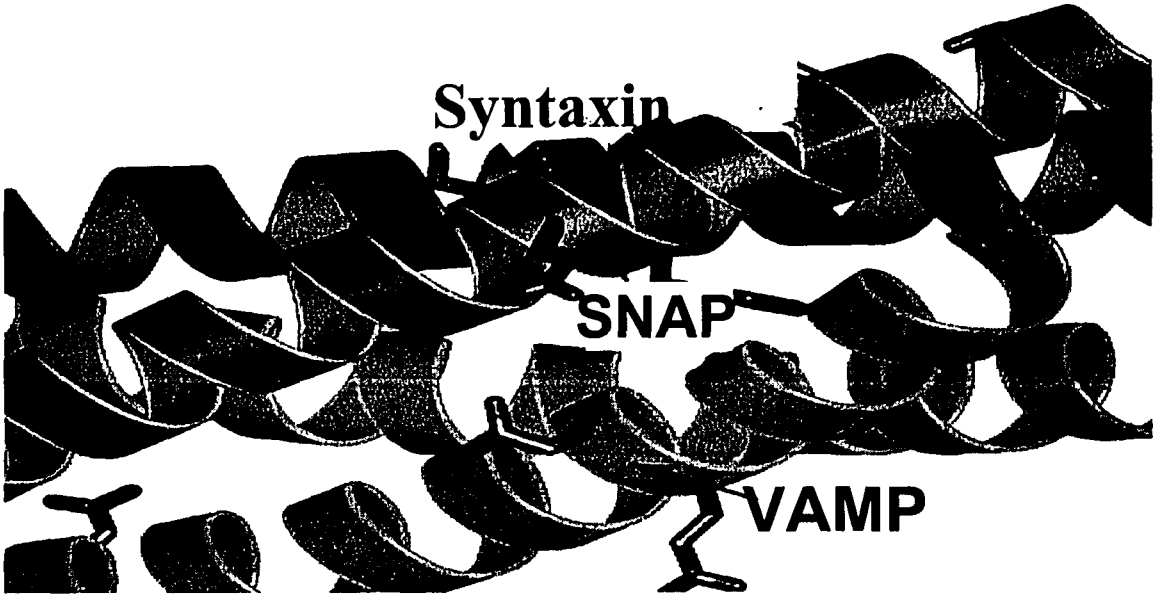

Figure 1.12 The protein backbone of the SNARE complex represented as a ribbon drawing.

The *trans*-SNARE complex (SNAREpin) is formed through the interaction of the v- and t-SNAREs. A SNARE complex includes three membrane proteins bound together, namely two t-SNAREs and one v-SNARE. Formation of this complex allows initiation of the fusion process by serving as a docking site for vesicles. Neuronal SNAREs acting at the synapse best illustrate vesicle targeting and fusion specificity. Blue, VAMP; red, syntaxin; green, SNAP-23 (Modified from: Fasshauer et al., 1998).

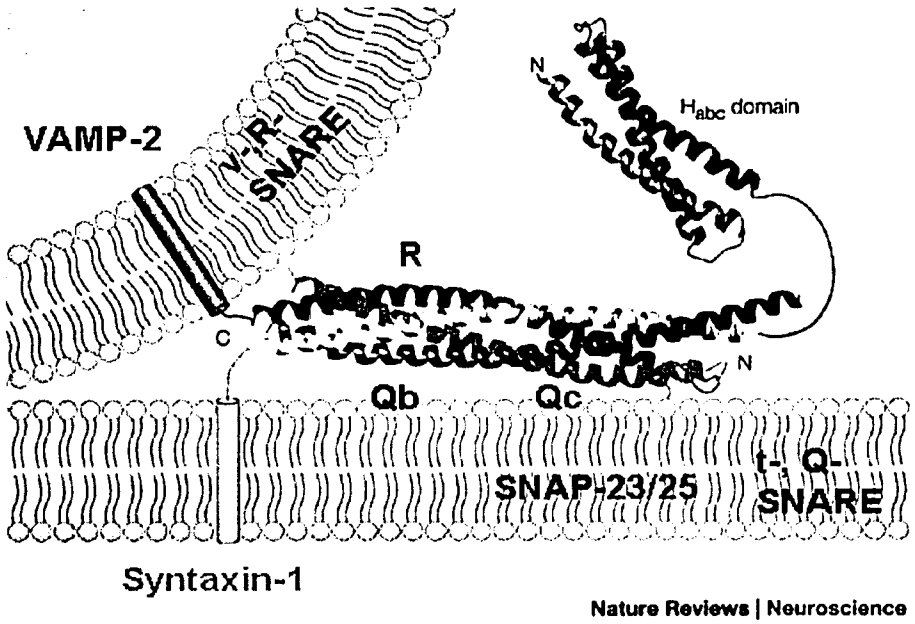

Figure 1.13 Model of the SNARE fusion complex.

The diagram represents the crystal structure of the SNARE core complex and the NMR (nuclear magnetic resonance) structure of the amino-terminal H_{abc} domain of syntaxin-1. H_{abc} (orange), VAMP-2 (red), syntaxin-1 (yellow), SNAP-25 amino-terminus (blue), SNAP-25 carboxyl terminus (green). The cylinders represent the transmembrane regions of VAMP-2 and syntaxin-1, which are inserted into the plasma membrane (Rizo and Sudhof, 2002).

2.3 The General SNARE Structure

The structural framework of SNAREs consists of small proteins from 100-300 amino acids in length (Fasshauer et al., 1998; Hong, 2005; Weimbs et al., 1997). All SNAREs have a conserved and homologous SNARE motif of about 60 amino acid domain involved in mediating core complex assembly during membrane fusion (Fasshauer et al., 1998; Jahn and Sudhof, 1999; Weimbs et al., 1997) (Figure 1.14). Many SNARE motifs are surrounded by short and variable sequences that do not form folding domains independently (Jahn and Sudhof, 1999). There are around 36 mammalian SNAREs with 31 showing only one SNARE motif (Hong, 2005). This motif is characterized by an anchoring C-terminal hydrophobic region and a variable N-terminal sequence (joining the polypeptide side to the cytoplasmic side). V-SNAREs usually have a single motif, whereas t-SNAREs can have either two or three polypeptides (Fukuda et al., 2000). The other 5 SNAREs interact with the C-terminal-anchored SNAREs (Vogel et al., 2000).

Of the SNAREs we are interested in, VAMP-7, syntaxin-3, syntaxin-4, and syntaxin-6 have extended coiled-coil N-terminal domains (Dietrich et al., 2004). There are five major types of N-terminal regions. Syntaxin/Qa is a three-helical bundle consisting of Ha, Hb, and Hc regions. In some syntaxin members, this Habc bundle can form a "closed" confirmation, folding back to interact with the C-terminal SNARE (Antonin et al., 2002; Dietrich et al., 2003; Fasshauer, 2003; Misura et al., 2001), whereas other syntaxin members the Habc bundle does not fold back and has an open conformation (Bracher and Weissenhorn, 2002; Dietrich et al., 2003; Dulubova et al., 2003; Dulubova et al., 2002; Yamaguchi et al., 2002). A third type of N-terminal

extension is characterized by the "longin" domain (Filippini et al., 2001; Rossi et al., 2004). This domain is characterized by a prolifin-like structure. VAMP-7 is considered to be a "longin" (Filippini et al., 2001; Rossi et al., 2004). VAMP-4 characterizes the fourth type of N-terminal domain extension. This extension targets VAMP-4 to the TGN. The fifth type of N-terminal extension is not well characterized (Burri et al., 2003; Dilcher et al., 2003; Nakajima et al., 2004). The SNAREs involved in this study are only associated with the first three types of N-terminal extension. As a group, the N-terminal extensions of SNAREs have a variety of regulatory roles, with many more to be defined (Reviewed in Hong, 2005).

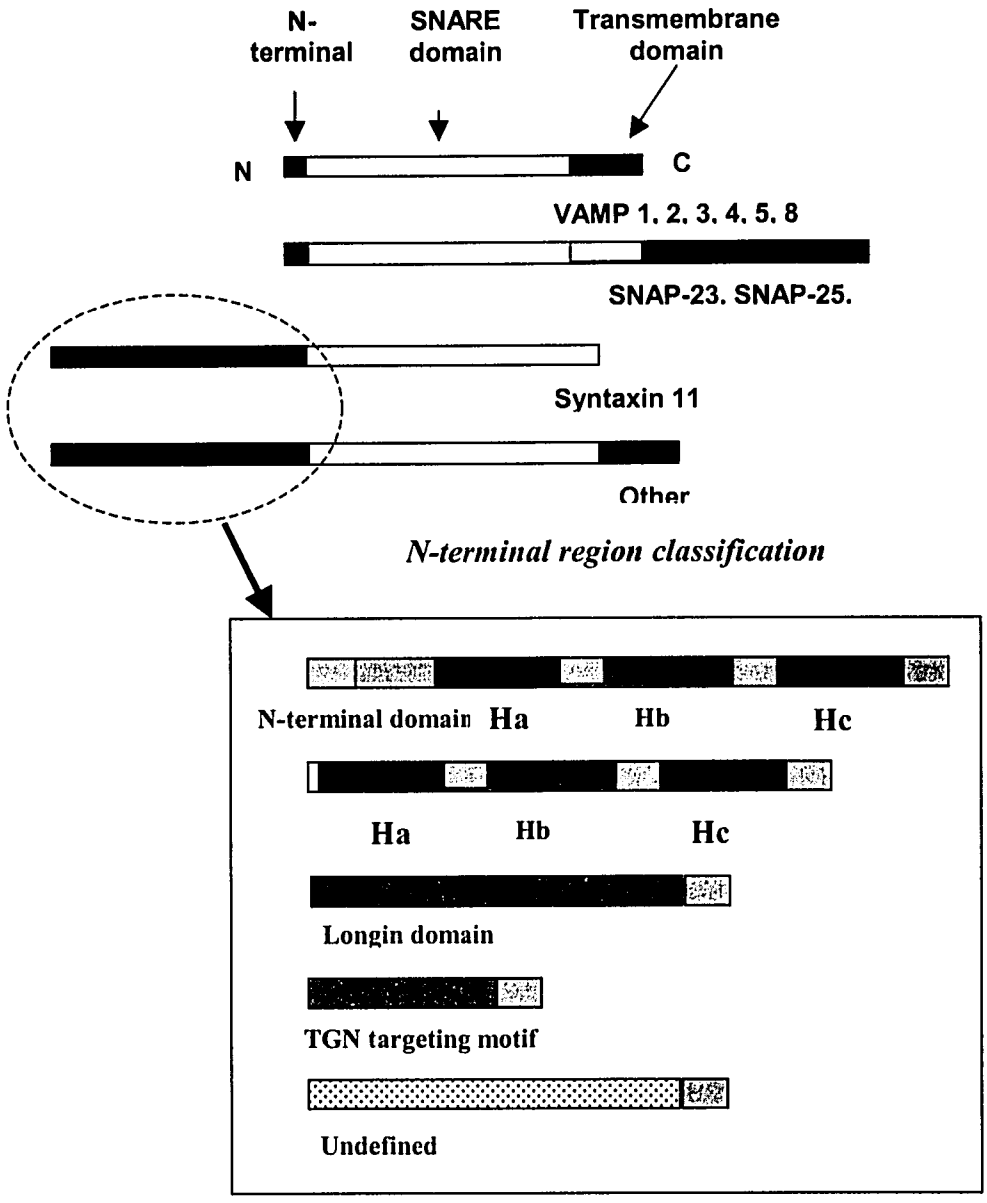

Figure 1.14 The general SNARE structure.

The SNARE motif is evolutionally conserved 60 residues present in all SNAREs. Approximately 36 mammalian SNAREs are known with the majority (31) are characterized by a C-terminal hydrophobic region functioning as a membrane anchor. Five major types of N-terminal regions exist. Syntaxin is three-helical bundle consisting of Ha, Hb, and Hc regions, which either have "closed" or "open" conformation. The "longin" domain characterizes the third type of N-terminal extension. This domain is characterized by a profiling-like structure. VAMP-7 is considered to be a "longin". The fourth type of N-terminal domain extension is characterized by VAMP-4 targeting VAMP-4 to the TGN. The fifth type of N-terminal extension is not well characterized. TGN-trans-golgi network (Adapted from: Hong, 2005).

2.4 Reclassification of SNARES

The original v- and t- SNARE classification was based on functionality (vesicle or target membrane protein), however, structurally SNAREs can also be classified as R or Q SNAREs according to the ionic residues interacting with the bundle (Fasshauer et al., 1998) (Table 1, Figure 1.15). Based on amino acid homologies four major subfamilies of SNARE proteins can be distinguished. SNARE subfamilies can be classified as S25N and S25C, based on either the C- or N- terminal of the SNAP-25 SNARE motif. The remaining SNAREs belong either to the syntaxin or VAMP subfamilies (Hong, 2005). Sixteen hydrophobic residues layers interact within the SNARE bundle (Fasshauer et al., 1998; Sutton et al., 1998). Classification is based on the ionic residue of the four-helical bundle at the zero ionic layer. The layers are organized from –7 at the N-terminal to +8 at the C-terminal with the 0 layer in the middle (Bock et al., 2001; Fasshauer et al., 1998). The 0 layer is mediated by three Gln (Q) residues (syntaxin, and the SNAPs) and one Arg (R) residue (VAMP) (Fasshauer et al., 1998; Sutton et al., 1998). Therefore, SNAREs are classified structurally as either Q or R SNARE based on the ionic residue at the 0 position (Bock et al., 2001; Fasshauer et al., 1998). Syntaxin is Qa SNARE, while S25N and S25C are Qb and Qc SNAREs, respectively. All VAMPs are R-SNAREs (Antonin et al., 2002; Bock et al., 2001; Chen and Scheller, 2001; Sutton et al., 1998). A four-helical bundle is formed when each of the four SNARE domains twist together forming the *trans*-SNARE complex (Sutton et al., 1998). Formation of this complex provides the energy to drive the fusion process (Fasshauer, 2003). The SNARE motif, therefore, is usually configured as Qa:Qb:Qc:R, where most Q SNAREs are t-SNAREs and most R-

SNAREs are v-SNAREs, with a few exceptions (Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000; Parlati et al., 2002).

Table 1- Alternative naming of SNAREs

SNARE Protein	Alternative Name	Classification (v-/R) (t-/Q)	Neurotoxin sensitivity
VAMP	synaptobrevin	v-/R	TeTx; BoTx B/D/F/G
VAMP-3	cellubrevin	v-/R	
VAMP-7	TI-VAMP	v-/R	TeTx insensitive
VAMP-8	Endobrevin	v-/R	TeTx insensitive
Syntaxin	HPC-1	t-/Q	BoTx C1
SNAP-23		t-/Q	

* TeTx, tetanus toxin
**BoTx, botulinum toxin;

Functionally	v-SNARE	t-SNARE
Sub-classification of t-SNARE	Heavy and light chain	Heavy and light chain
Structurally according to the central residue of SNARE motif (0 layer)	R-SNARE	Q-SNARE
Sub-classification of Q-SNARE	Qa, Qb, and Qc	

Figure 1.15 Classification of SNAREs.

SNAREs were originally classified as v- (on vesicles) or t- (target) components. A t-SNARE has one heavy chain and two light chain SNARE domains. The two light chains can come from one or two proteins. Based on the zero ionic residue of the SNARE bundle, SNAREs have been reclassified as Q-SNARE (Gln residue) and R-SNARE (Arg residue). Q-SNAREs can be divided into Qa, Qb, and Qc SNAREs based on the amino acid sequences (Hong, 2005).

2.5 Rab Proteins

In exocytosis, regulation of the plasma membrane and secretory granule fusion is thought to involve small G proteins belonging to the Rab family (Roa et al., 1997). Rab proteins are part of the Ras-like superfamily of proteins (Martinez and Goud, 1998; Novick and Zerial, 1997). Rabs are the largest family of small monomeric GTPases that contribute to specificity of vesicular transport (Fischer von Mollard et al., 1994; Zerial and McBride, 2001). The role of Rab proteins in higher eukaryotes stems from work on the yeast, *Saccharomyces cerevisiae* (Lledo et al., 1994). Biochemical studies on Rab proteins have shown that Rabs are crucial components of intracellular membrane trafficking steps in eukaryotes (Fischer von Mollard et al., 1994). Through studies with expressed sequence tags (EST) and the human genome is now thought that as many as 63 Rab proteins are present in humans and 11 Rabs in yeast including Yptp/Sec4p, key regulators of the secretory pathway (Lledo et al., 1994; Zerial and McBride, 2001). Each of the Rab proteins identified in mammals is localized to a particular compartment in the membrane and act upstream of the fusion machinery (Masuda et al., 2000) (Figure 1.16). Rabs are distributed on the cell membrane and at least one Rab protein is found in every organelle. Rabs regulate intracellular organelle transport by matching appropriate v- and t-SNAREs. Rab distribution is to specific intracellular compartment. A study by Zerial and McBride, 2001 investigated the possible mechanisms through which Rab proteins regulate SNARE function determined from interactions at the molecular level between components of the SNARE machinery and Rab effectors (Zerial and McBride, 2001).

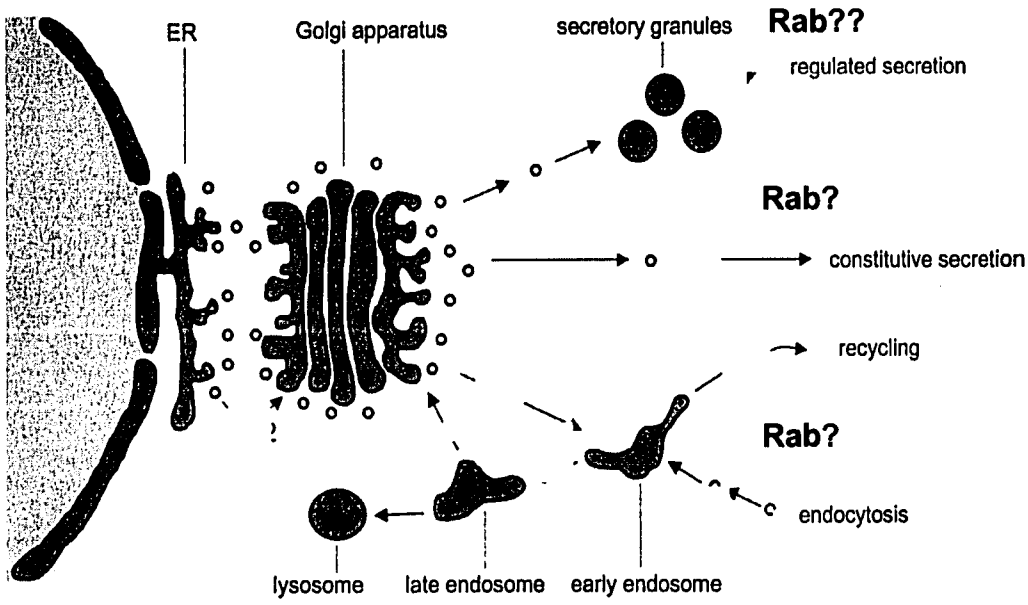

Figure 1.16 Localization of Rab protein to various intracellular compartments.

Rabs localization can be to target compartments (e.g. Rab5) or to donor compartments (e.g. Rab3). Therefore, Rabs are involved in various steps of trafficking within the cell. Rab proteins have not yet been fully identified in particular traffic steps (Adapted from: Tuvim et al., 2001).

Rab 1, 2, 3, 4, 5, 8, and 9 have all been linked to exocytosis (Lledo et al., 1994). Fischer von Mollard *et al.*, 1994 showed that Rabs might be involved in a step upstream for exocytosis, but downstream from docking. It was later shown that Rabs might be involved in budding of vesicles and interactions with the cytoskeleton in addition to tethering/docking of vesicles to target membranes and fusion (Zerial and McBride, 2001). One regulatory pathway may control all vesicle transport since Rab proteins were found to have several functions (Zerial and McBride, 2001).

2.6 Involvement of SNARE and Rab Proteins in Secretion of Non-neuronal Cells

The function of SNARE and Rab proteins intersects. However, there is little evidence to support direct interaction of SNAREs and Rabs (Jahn and Sudhof, 1999). Rab tethering and docking and membrane fusion through SNARE molecules are critical in understanding vesicular transport. Orderly membrane trafficking occurs by high selectivity of transport vesicles therefore, many interactions with potential targets occurs before the correct one is found.

Target membranes display receptors specific for markers on the surface of the vesicles, thus ensuring specificity of fusion. SNAREs and Rab GTPases control recognition by allowing specificity to target membranes for fusion and by regulation of docking and tethering to targets on the plasma membrane (Zerial and McBride, 2001). Once primed, a *cis*-SNARE complex forms in the Rab domain leading to tethering and docking followed by the formation of a *trans*- SNARE complex between the target SNARE and the vesicle. *Cis*-SNARE selectivity occurs at the functional site after Rab effectors interact at a molecular level within the Rab domain leading to linkage of Rab

effectors with SNARE machinery components. The selective environment for vesicle tethering is due to assembly of SNARE machinery and Rab effectors into specific compartments. The first event in membrane tethering mediated by Rab proteins immediately precedes SNARE pairing to ensure that the fusion event occurs with precision between the bilayers (SNAREpins). Both Rabs and effectors trigger SNARE protein release and concentrate tethered vesicles to their targets (Zerial and McBride, 2001). Sogaard *et al.*, 1994, showed that a SNARE complex assembly requires Rab proteins. Rabs, therefore, act as catalysts in SNARE complex formation, but do not appear to be part of the docking complex.

Little work has been done with respect to cytotoxic lymphocytes and SNARE machinery. Based on finding from other cell types, it is thought that cytotoxic lymphocytes express vesicular SNAREs (VAMPs, associated with vesicles/granules) and target SNAREs (syntaxin and SNAP-23, associated with the plasma membrane fractions), which are thought to be involved in the regulation of vesicular docking and secretion of granule contents. Rab-related GTPases might be associated with specific processes involved in specificity of membrane fusion (Figure 1.17). This research study has investigated various molecular processes of vesicular trafficking and mediator release in cytotoxic lymphocytes during exocytosis. By determining isoforms of Rab and SNARE proteins and determining location and function, understanding the mechanisms used by CTLs and NK cells to kill target cells may be better achieved.

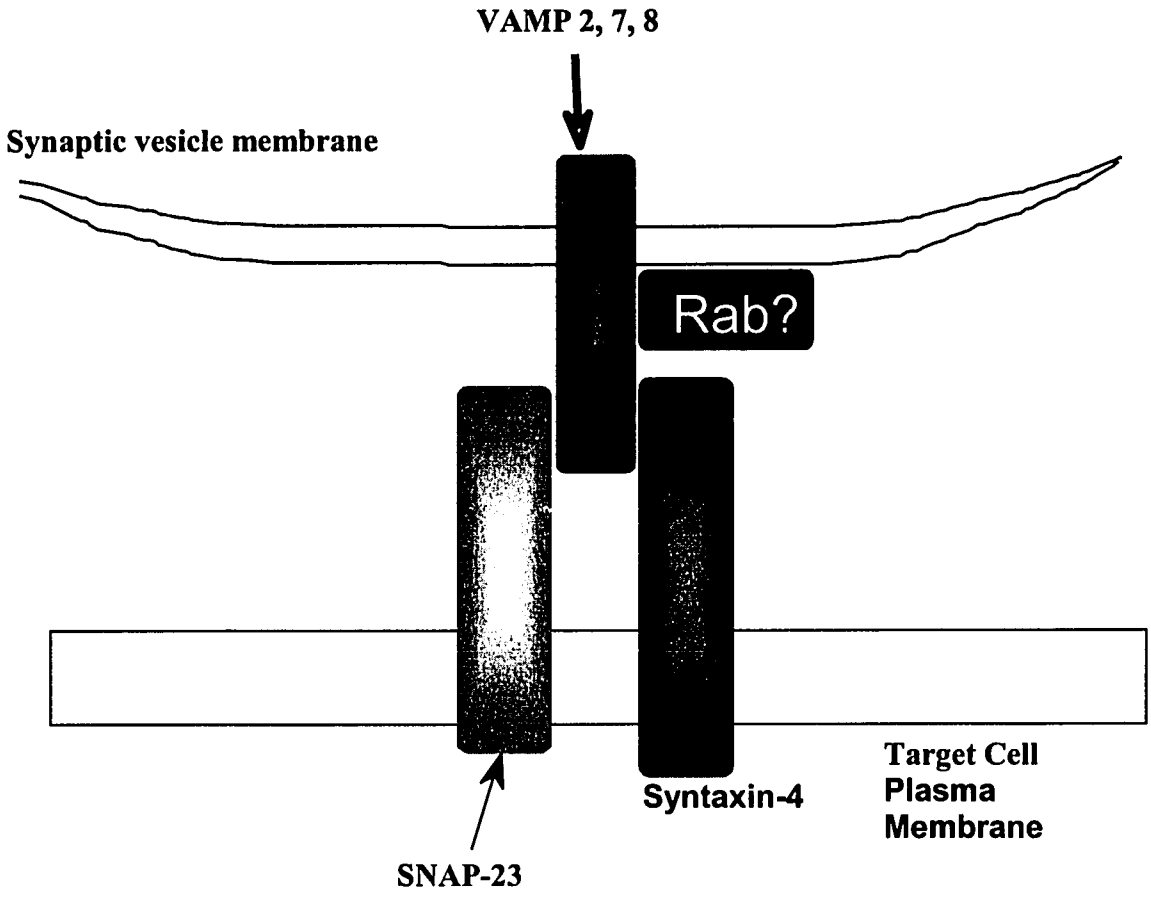

Figure 1.17 A putative docking and fusion complex of non-neuronal cells.

The overall fusion complex involves a v-/R- SNARE (VAMP 2, 7, 8) and two t-/Q-SNAREs (SNAP-23 and syntaxin-4). An undetermined Rab protein serves to match the appropriate v-/R- and t-/Q- SNAREs together.

II. Hypothesis and Study Objectives

Based on the above as well as preliminary data generated, the hypothesis is GrB containing cells (CTLs and NK cells) express SNAREs and Rab proteins, which are essential components of lymphocyte-mediated exocytosis.

Study Objectives

Based on literature and reports of SNAREs identified in hematopoietic cells, the anticipation is that certain SNARE isoforms are common to all cells of the hematopoietic lineage and therefore, will also be present in CTLs and NK cells. Additionally, SNARE isoforms will be identified that are specific in expression or localization to CTLs and NK cells. This project has two main aims:

Aim 1: Identification of the isoforms of SNARE and Rab proteins expressed in GrB^+ cells

In order to determine the isoforms of SNAREs expressed in GrB-containing cells, human $CD8^+$ CTLs and the natural killer (NK) cell line, YT-Indy (see chapter 2) were examined. Specific primers for SNARE and Rab molecules were used to determine the presence of message encoding for v-SNAREs; VAMP-1, VAMP-2, VAMP-3, VAMP-7, VAMP-8, and t-SNAREs; syntaxin-3, syntaxin-4, syntaxin-6, SNAP-23, as well as upstream proteins thought to be associated with regulating SNARE expression and phosphorylation, Rab4a, Rab5a, and Rab27a. This was determined using reverse transcription-polymerase chain reaction (RT-PCR) followed by gel electrophoresis on a 2% agarose gel stained with ethidium bromide. The presence of mRNA was determined for the v and t-SNAREs. .

Confirmation of protein isoform expression was determined by Western blot analysis. Specific antibodies for SNARE molecules were used to determine the presence of protein expression in CTLs and YT-Indy for the SNARE isoforms, VAMP-2, VAMP-7, VAMP-8, syntaxin-4, syntaxin-6 and SNAP-23, based on availability of specific antibodies.

The results from these two experiments indicate that GrB-containing cells express both mRNA and protein for various isoforms of SNAREs, and Rabs, suggesting that GrB-containing cells possess the necessary secretory machinery found in conventional secretory cells, which may be important in regulation of GrB release.

Aim 2: Colocalization SNARE proteins with GrB^+ granules in CTLs and NK cells

Confocal microscopy and immunocytochemistry determined if colocalization between SNARE proteins and GrB^+ granules occurred in CTLs and YT-Indy. Activated human $CD8^+$ CTL clones and YT-Indy were stained with anti-GrB and anti-v-SNARE antibodies and the lysosomal marker, CD63. Colocalization of v-SNAREs to GrB would indicate that these two proteins coexist and function as part of CTL mediated killing.

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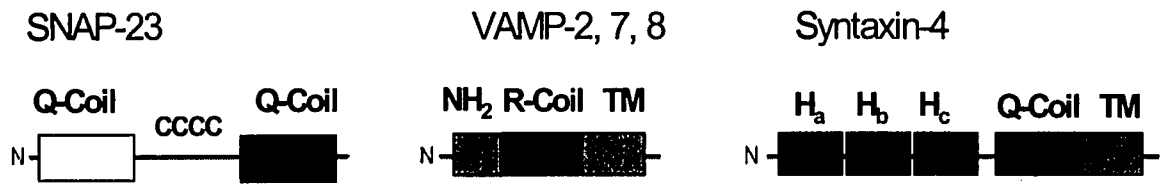
Chapter 2

I. Methodology

1.1 Reagents

SNARE Antibodies

Sodium-azide (NaN_3)-free mouse monoclonal antibodies (mAb) (IgG_1) specific for the cytoplasmic domains of human recombinant syntaxin-4 (aa 1-280), syntaxin-6 (aa 1-136), and mouse monoclonal anti-VAMP-2 antibody (C169.1, rat VAMP-2 aa 2-17) raised against a synthetic peptide, CSATAATVPPAAPAGEF corresponding to the N-terminus of rat VAMP-2 were obtained from BD Pharmingen (San Diego, CA, USA). Rabbit anti-SNAP-23 antiserum, raised against the synthetic C-terminus peptide sequence, DRIDIANARAKKLIDS was obtained from Synaptic Systems GmbH (Göttingen, Germany). Affinity-purified rabbit anti-SNAP-23 was a generous gift from Dr D. Castle (University of Virginia Health Sciences Center, Charlottesville, VA). Mouse monoclonal antibody (mAb) to human TI-VAMP (VAMP-7) (clone 158.2 directed against amino acids 119-188) (Muzerelle et al., 2003) and affinity purified anti-human VAMP-8 (TG15) rabbit IgG (N-terminus MEEASGSAGNDRVRN) (Muzerelle et al., 2003; Paumet et al., 2000) were kind gifts from Dr T. Galli (Membrane Traffic and Neuronal Plasticity Group, INSERM U536, Institut du Fer-a-moulin, Paris, France) (Paumet et al., 2000). Affinity purified anti-human VAMP-8 rabbit IgG was also obtained from Abcam (Abcam, Inc. Cambridge, MA). The SNARE antibodies used and their binding sites are summarized in figure 2.1.



Antibody	Region	Recognizes
Anti-VAMP-2	NH_2 domain	Monomeric protein
Anti-VAMP-7	NH_2 domain &coil	Monomeric protein
Anti-VAMP-8	NH_2 domain	Monomeric protein
Anti-syntaxin-4	H_{abc}, coil	Monomeric & complexed protein
Anti-syntaxin-6	H_{abc}, coil	Monomeric & complexed protein
Anti-SNAP-23	C term. coil	Monomeric & complexed protein

Figure 2.1 SNARE domains and antibody recognition sites.

Antibodies used and their recognition regions are shown. The amino-terminal domain of syntaxin forms a three helical bundle (red) that binds to its carboxyl-terminal coil domain (purple), forming a closed conformation. One domain of syntaxin (t/Q-SNARE) combines with two SNAP-23 (t/Q-SNARE) and one v-/R SNARE (either VAMP-2, 7, or -8) to form the four-helix SNARE complex (Modified from: Chen and Scheller, 2001).

Other antibodies

Biotin conjugated anti-granzyme B was a gift from Dr. R.C. Bleackley, (University of Alberta, Edmonton, Canada) originally from Research Diagnostics Inc. (Pleasant Hill Road Flanders NJ 07836 USA). Biotin conjugated mouse IgG isotype control was obtained from Research Diagnostics Inc. (Pleasant Hill Road Flanders NJ 07836 USA). Streptavadin was obtained from Caltag Laboratories (Burlingame, CA). NeutrAvidin Biotin-Binding Protein was purchased from Pierce Biotechnology (Rockford, IL). Goat anti-mouse Alexa 594 was purchased from (Molecular probes, Eugene, Ore). Rhodamine Red-X-conjugated affinity purified goat anti-mouse IgG and Rhodamine-Red-X-conjugated affinity purified donkey anti-rabbit IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). BODIPY FL-labeled goat anti-mouse antibodies and Prolong antifade kit were purchased from Molecular Probes (Eugene OR, USA). Mouse anti-CD63 mAb (IgG_1) was purchased from BD Pharmingen. Mouse IgG_1 and purified rabbit IgG were obtained from R&D Systems (Minneapolis, MN, USA) and Cedarlane Laboratories (Hornby, ON, Canada). Donkey serum was obtained from SIGMA-Aldrich,Inc.(SaintLouis,MO).

Other reagents

Components of phosphate buffered saline (PBS); 0.14mM NaCl, 3.6 mM KCl, 2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$-H_2O, 0.5 mM $MgCl_2$-$6H_2O$, pH 7.2, were filter sterilized as was Tris buffered saline (TBS); 50 mM Tris base, 150mM NaCl to 1L H_2O, pH 7.5.

1.2 Cell Lines

The human CTL clones, $hCD8^+$ were a gift from Dr. R. C. Bleackley (University of Alberta) and were cultured in RPMI 1640 (SIGMA-Aldrich, Inc.) supplemented with 8% FBS, 2 mM L-glutamine, 1mM sodium pyruvate, $5X10^{-5}$ M β-mercaptoethanol, 40U/mL penicillin/streptomycin, 25mM HEPES, and $NaHCO_3$. These cells were also supplemented with 10U/mL of recombinant human IL-2 (rhIL-2) R&D Systems (Minneapolis, Minn.) YT-Indy cell line were also a gift from Dr. R.C. Bleackley and cultured in RPMI 1640 (SIGMA-Aldrich, Inc.) supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 40U/mL penicillin/streptomycin, 0.1 mM non-essential amino acids, 25mM HEPES, and $NaHCO_3$. Stimulation/target cells, RPMI 8666 were a gift from Dr. R.C. Bleackley and cultured in RPMI 1640 (SIGMA-Aldrich, Inc.) supplemented with 8% FBS, 2 mM L-glutamine, 1mM sodium pyruvate, $5X10^{-5}$ M β-mercaptoethanol, 40U/mL penicillin/streptomycin, 25mM HEPES, and $NaHCO_3$. All cells were maintained in an incubator at 37°C with 5% CO_2.

1.3 Culturing $CD8^+$ Clones

$CD8^+$ clones were counted at peak density. RPMI 8666 stimulatory/target cells were harvested at a density, which doubled that of the $CD8^+$ clones. These RPMI 8666 cells were irradiated at 5000 rads for 2 h, spun down and resuspended in fresh RPMI media. The irradiated RPMI 8666 cells were added to the $CD8^+$ clones in a 2:1 ratio and grown in co-culture for 3 days in the presence of rhIL-2. On the third day the cells were layered on Ficoll-paque (from Amersham Biosciences PQ, Canada) in a ratio of 3:1 (cells: Ficoll) to remove irradiated RPMI 8666 cells.

1.4 RT-PCR

1.41 Primer Design

Primers for SNAREs and the housekeeping gene, GAPDH, were confirmed against GENBANK known sequences (National Center of Biotechnology Information (NCBI)). Primer sequences and expected product sizes are located in Table 2. Amplified products were subcloned and sequenced in our lab during previous studies on human neutrophils and eosinophils. Briefly, PCR products were subcloned into the pCR™ 2.1 plasmid vector using the TA cloning kit (Invitrogen Corportation, Carlsbad, CA) and double-stranded plasmid DNA isolated with the Wizard Plus Mini-Prep kit (Promega Corporation, Madison, WI) as previously described (Lacy et al., 1999). Plasmid DNA containing the PCR product insert was sequenced using M13 forward and reverse primers and carried out on an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA).

Table 2- Primer Sequences for Amplification of SNARE Proteins Using RT-PCR

SNARE isoforms	Primer sequences	Number of cycles	Annealing Temp (°C)	RT-PCR product size (bp)
VAMP-1[5]	F: 5'-CAGTTCCGTCCACTTCAGC-3' R: 5'-CCTCCTTAGCTTGGCAGCAC-3'	40	60	356
VAMP-2[4]	F: 5'-ATGTCTGCTACCGCTGCCACG-3' R: 5'-AGAGCTGAAGTAAACTATGATGATG-3'	30	50	348
VAMP-3[6]	F: 5'-ATGTCTACAGGTCCAACTGCTG- 3' R: 5'-GCTGGTTCTTCATGAAGAGACA-3'	40	60	315
VAMP-7[1]	F: 5'-AGACTGAAGCCATGGCGATT –3' R: 5'-CTATTTCTTCACACAGCTTGGC-3'	40	60	684
VAMP-8[2]	F: 5'-GGCGAATTCACTTACTGACC –3' R: 5'-GTCTCTCCAGCCCACTCTAA –3'	40	60	587
Syntaxin-3	F: 5'-GTATCATTCTCTCTGCACCG-3' R: 5'-CATGTCGTGAAGCTCCTTGA-3'	40	61	462
Syntaxin-4[7]	F: 5'-ATGCGCGACAGGACCCACGAGCTG-3' R: 5'-TTATCCAACCACTGTGACGCCAATGAT-3'	40	61	894
Syntaxin-6[3]	F: 5'-CCCTTCTTTGTGGTGAAAGG-3' R: 5'-CTGGGAACATGTTCTTCAGCA-3'	40	57	549
SNAP-23[8]	F: 5'-CTGGGTTTAGCCATTGAGTCTCAGG-3' R: 5'-GGTGTCAGCCTTGTCTGTGATTCG-3	30	62	498
Rab3D[9]	F: 5'-TACCGCCATGACAAGAGGAT-3' R: 5'-AAGCTCATGACCGTCAGTGT-3'	40	60	730
Rab4A[10]	F: 5'-AACAGATGCCCGAATGCTAG-3' R: 5'-GGCTACAGGACATACAGTAC-3'	40	60	622
Rab5A[11]	F: 5'-GGATAGAGCTGGTCAAGAAC-3' R: 5'- AGGTGACTACTGTACCAGTG-3'	40	60	773
Rab27A[12]	F:-5'-GGACCAGAGAGTAGTGAAAG-3' R:-5'-CAGGTGACAGTACCCTCATT-3'	40	60	573

1-NIH Genebank Access No. NM_005638; 2-NIH Genebank Access No. NM_003761; 3- NIH Genebank Access No. NM_005819;4-NIH Genebank Access No. NM_014232.1; 5- NIH Genebank Access No. NM_014231; 6- NIH Genebank Access No. NM_004781; 7- NIH Genebank Access No. AF026007; 8-NIH Genebank Access No. V55936; 9- NIH Genebank Access No. NM_004283; 10- NIH Genebank Access No. BC004309; 11- NIH Genebank Access No. NM_004162; 12- NIH Genebank Access No. NM_004580.

Sequencing of products was previously performed using a BLAST sequence similarity search over the website of the National Center for Biotechnology Information (NCBI) in studies on human eosinophils and neutrophils. All other primers were designed using the Primer3 design program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

1.42 RNA Extraction

Total RNA extracted ($6X10^6$ cells minimum/extraction) was subjected to RT-PCR with specific primers for SNARE or Rab proteins. Total RNA was extracted from samples using the QIAGEN QIAShredder and RNeasy MiniKit (QIAGEN Inc, Mississauga, ON). Approximately 1.0 μg of total RNA was used for the RT reaction. The RNA optical density was determined by a spectrophotometer at 260nm. Samples were prepared by adding 298μl of ddH2O into an eppindorf tube followed by addition of 2μl of RNA sample and mixed well. The concentration of RNA was determined by μg/μl of sample RNA = OD260 x 100 x 40/1000 or with OD x 8.0. Samples were stored at -80°C until needed. A more detailed materials section can be found on page 129.

1.43 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The RT-PCR reaction was carried out with Moloney murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies, Grand Island, NY) or Superscript II reverse transcriptase from Invitrogen (Mississauga, ON). Reverse transcription was carried out by adding µg total RNA to 1µl oligo-(dT) (500µg/ml) and topped to 11µl with RNase-free water. The mixture was heated for 10 min at 70° C, following which 7.5µl of RT master mix was added per reaction (4µl 5X First stand buffer (Gibco BRL Life Technologies, Grand Island, NY), 2µl 0.1 M DTT, 1µl mixed dNTPs (10 mM), 1µl RNase-free water). The reactions were incubated for 37° C for 1 hr following the addition of 1µl of reverse-transcriptase (200 units). Heating at 70°C for 10 min terminated reactions. The cDNA generated was stored at –20° C or used immediately for PCR reactions.

PCR reactions were carried out in 20μl volume according to the following protocol. Stock master mix was prepared to contain 2μl 10X PCR buffer, 0.4μl 10mM dNTPs, 0.6μl $MgCl_2$, 13.9μl sigma H_2O per reaction tube. To each reaction tube, 2μl of cDNA was added followed by 0.5μl of each forward and reverse primer (20pmol). A volume of 0.5μl of Taq polymerase (Gibco BRL Life Technologies, Grand Island, NY) was added immediately before amplification. Reactions were carried out in a PTC 100 Programmable Thermal Controller (M-J Research, Watertown, Mass).

Samples were run along side documented control cells (Advani et al., 1998; Hibi et al., 2000; Logan et al., 2002; Martin-Martin et al., 2000; Martin-Martin et al., 1999; Ralston et al., 1994). Products were electrophoretically separated on a 2% agarose gel, stained with ethidium bromide (EtBr) and visualized by ultraviolet light. The product size was determined using a 1Kb DNA ladder (Invitrogen, Mississauga, ON). CaCo2 cells were provided by Dr. Glen Armstrong (University of Calgary). Detailed PCR reaction temperatures and steps can be found on page 130 of the appendix.

1.5 Western Blot Analysis

1.51 Preparing Lysates and Protein Assay

Cells were collected at peak density and resuspended at a minimum concentration of 6×10^6 cells per lysis. Cells were resuspended in RIPA lysis buffer (see appendix) and kept on ice for 30 min. Nuclei were removed by centrifugation at 7,000Xg for 10 min and lysates stored at –80°C until use. Lysates of human platelets and rat brain were used

as positive controls for SNARE protein expression (Advani et al., 1999; Chen et al., 2000; Feng et al., 2002; Polgar et al., 2002; Sesack and Snyder, 1995). Whole rat brain was homogenized, using a Dounce homogenizer, in 3 ml of RIPA buffer containing 5μg/ml protease inhibitor cocktail (leupeptin, aprotinin and TAME, Sigma). Homogenate was incubated on ice for 30 min and nuclei/tissue debris removed by centrifugation at 7,000Xg for 10 min. Homogenates were aliquoted and the supernatant was stored at – 80°C. Platelet lysates were obtained from peripheral blood (50 ml) of atopic donors, and subjected to centrifugation at 300g for 10 min. Plasma-rich supernatant devoid of cells was harvested and centrifuged at 1800Xg for 5-8 min. Platelet-rich pellet was resuspended in 1 ml 0.25 M sucrose, 10 mM HEPES, pH 7.4, and 1 mM EGTA containing 0.1% Triton X-100. The samples were centrifuged at 8, 00xg for 10 min at -80°C. Protein contents of lysates were determined by using the DC pro assay (Bio-Rad Laboratories, Canada). A 1% BSA sample was used for the standard curve diluted in RIPA buffer. Working stock solution was prepared as per package instructions, incubated for 30 minutes at 37 ° C, and read on a plate reader at 690nm.

1.52 Western Blot Analysis

Cell lysates from human CTLs and YT-Indy were resuspended in an equal volume of 3XSDS loading buffer (New England Biolabs, Beverly, MA, USA) along with Precision Plus Protein Standard (Biorad, Hercules, CA, USA) and subjected to electrophoretic separation on 10% acrylamide gels (SDS-PAGE) between 100-120 volts for 3hrs on the basis of a previous report (Logan et al., 2002). Protein from electrophoresed gels were transferred to Immobilon PVDF membranes (Millipore, Bedford, MA, USA) using a semi-dry transfer (BioRad Laboratories) system for 50 min

at 15 V. Immobilon blots were blocked with 5% skim milk powder w/v in TBS+0.5 %Tween-20 (TBS-Tw) for 1hr at room temperature with gentle rocking. Primary antibodies were diluted in 5% skim milk powder and TBS-Tw (with the exception of TI-VAMP, which was diluted in TBS-Tw only) and incubated overnight at 4°C with gentle rocking. Antibody dilutions were as follows: SNAP-23 mAb: 1:5000; syntaxin-4 mAb: 1: 5000 (50ng/ml); syntaxin-6 mAb: 1:1000 (250ng/ml); VAMP-8 was used at a concentration of 1: 1000; VAMP-7 (TI-VAMP) mAb (1:1000) and VAMP-2 mAb (1:5000) (50 ng/mL). Platelet lysates were prepared as a positive control for SNAP-23, syntaxin-4 and VAMP-8. Rat brain homogenates were prepared as a positive control for syntaxin-6, VAMP-2 and VAMP-7, respectively. Blots were quickly rinsed in TBS-Tw followed by 2X10 min washes. Detection was with peroxidase-conjugated AffiniPure F (ab') fragment anti-mouse IgG (H + L) (1:5,000) or peroxidase-conjugated AffiniPure Goat anti-Rabbit IgG, F (ab') fragment specific (1:5,000) (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) secondary antibodies. Blots were once again rinsed quickly in TBS-Tw followed by 2X10 min washes. Chemiluminescence has was developed by addition of SuperSignal substrate solution (Pierce, Rockford, IL) for 1 min and exposed to Kodak film or Hyperfilm (Amersham Biosciences) ranging between 5 minutes to overnight. Band sizes were estimated by comparing with molecular weight standards. A detailed description of materials can be found on page 131 of the appendix.

1.6 Immunocytochemistry and Confocal Laser Scanning Microscopy

1.61 Single Labeling

Cytospin preparations of cells were washed twice and resuspended in colour-free RPMI supplemented with 20% FBS. Cells were resuspended at 30, 000 cells/100uL of RPMI. A cell suspension of 100μL was added to the cytospin funnels and spun at 8000Xg for 5 min on Cytospin 2 (Shandon, Astmoor, Runcorn, U.K.), as previously optimized (Lacy et al., 2003). Slides were air dried, and wrapped in aluminum foil and stored at –20°C. When needed, cytospins were thawed at room temperature for 30 min and fixed in neutral-buffered formalin for 5 min. A 4% solution of donkey serum was prepared in 1X PBS to which was added to prepare a 10% solution of neutravadin. After fixation, the cytospins were washed well by rinsing with 1XPBS followed by a 5 min PBS bath. Cytospins were permeabilized for 5 min in 0.05% triton X-100 at room temperature. Slides were rinsed with PBS. Depending on the antibodies used, cytospins were blocked with either 4% donkey serum or 10% neutravadin (for biotin-conjugated antibodies) for 1 hr at room temperature, followed by a PBS wash. Purified rabbit IgG1 was used as a negative control for VAMP-8, while mouse IgG1 was used as a control for VAMP-7 and CD63, and biotin conjugated mouse IgG1 was used as a negative control for biotin conjugated GrB. Antibody dilutions were prepared in 4% donkey serum as shown in Table 3.

Table 3- Immunocytochemistry Dilutions

Antibody	Stock concentration	Dilution
VAMP-7	10mg/mL	1:100
VAMP-8	ND[1]	1:100
CD63	0.5mg/mL	1:70
GrB	0.5mg/mL	1:200
mIgG1	0.5 mg/mL	1:50
rIgG1	2 mg/mL	1:200
mIgG1:biotin	1 mg/mL	1:100
Goat anti-mouse Alexa 594	2mg/mL	1:1200
StreptAvadin-FITC	4-5 mg/mL	1:400
Donkey anti-rabbit Rhodamine Red	10ug/mL	1:150
Goat anti-mouse Rhodamine Red	10ug/mL	1:150
BODIPY-FL	20 μg/ml	1:50

1-ND: no data

Slides were incubated for 1 hr with the primary antibody dilutions (VAMP-7, VAMP-8, CD63, and GrB) and rinsed with PBS. Immunoreactivity was detected using secondary antibodies (Alexa 594 (VAMP-7), donkey anti-rabbit rhodamine red–X-conjugated (VAMP-8), or goat anti-mouse rhodamine-red-X-conjugated (CD63) and streptavadin-FITC (GrB-biotin) and incubated in the dark at room temperature for 1 hr. Cells were rinsed well with PBS followed by a PBS bath for 15 min in low lighting. Slides with the same species of primary antibodies (VAMP-7/CD63) were incubated for 1 hr at room temperature with 10μg/ml goat anti-mouse F (ab') fragments in order to reduce any non-specific binding. At this point, slides were rinsed either mounted (single labeling) or prepared for double labeling.

1.62 Double Labeling

Second primary antibodies (GrB: biotin, or CD63) were added to double-labeled slides and incubated at room temperature in the dark for 1 hr. This was followed by a PBS rinse, addition of the second secondary antibodies (Streptavadin-FITC or BODIPY FL) and incubation in the dark for 1 hr. Cytospins were then washed well with PBS and put into a PBS bath for 15 min. Slides were wiped and stained with 4',6'-diamidino-2-phenylindole nuclear stain (DAPI) (1:500) for 5 min and rinsed well in PBS. Slides were mounted with 1 drop of ProLong Antifade Gold kit (Molecular Probes) and prepared as per supplier instructions. Slides were left to dry and stored in the dark at room temperature. Images were collected as previously described (Lacy et al., 1998). Briefly, confocal analysis was carried out on labeled cells using a 40x objective on a Zeiss

confocal laser-scanning microscope (Carl Zeiss Microimaging, Thornwood, NY). The Molecular Dynamics Confocal Imaging System 2001 (Molecular Dynamics, Sunnyvale, Calif) and Image Space 3.2 software were used for detection and imaging of immunofluorescence (Lacy et al., 1998).

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Chapter 3

I. Results

1.1 Introduction

Cytotoxic T-cells (CTL) and natural killer cells (NK) destroy cells that express altered-self, including virus infected, tumour, and allogenic cells. Both are efficient killers with a high degree of specificity for target cells (Fleischer, 1986; Smyth et al., 2005). The main pathway that CTLs and NK destroy their targets, is via caspase-dependent apoptosis through the granzyme-B (GrB)-perforin pathway (Smyth et al., 2005). These two molecules are cytotoxic and their release is highly regulated.

While conventional secretory cells use separate organelles for storage and release of granule contents, CTLs and NK cells use a single type of organelle, which performs this dual function, namely the secretory lysosome (SL). Secretory lysosomes express membrane proteins, lysosomal hydrolases (β-hexosaminidase) and a number of lysosomal markers such as CD63, (Griffiths, 1996). Regulated secretion of SLs is necessary in CTLs and NK cells because they store cytotoxic proteins, perforin and GrB (Bossi and Griffiths, 1999; Thompson and Allison, 1997). Overlap between secretory and lysosomal markers (CD63) in mature CTLs and NK cells suggests that the majority of lysosomes are secretory in nature. My study is concerned with docking and fusion portion of secretion and the putative proteins and machinery involved in these two processes, particularly the SNARE proteins (Blott and Griffiths, 2002; Stinchcombe and Griffiths, 2001).

SNAREs (soluble NSF [N-ethylmaleimide-sensitive factor] attachment proteins receptors {SNAP receptors}) (Rothman, 1994; Sollner et al., 1993) have been shown in other cells of the haematopoietic lineage to be involved in regulating docking of membrane-bound vesicles during exocytosis. SNAREs are representative of a group of membrane-bound proteins categorized into two groups, v/R-SNAREs (vesicle specific receptors) and Q/t-SNAREs (target membrane specific receptor) (Rothman, 1994; Sollner et al., 1993). Through the formation of NSF and the SNAP complex, v-SNARE/t-SNARE interactions occur where contact is established between vesicles and the target membrane (Jahn and Sudhof, 1999). In neuronal cells, studies show that a SNARE complex requires a minimum of three membrane proteins complexed together in order to bind vesicle associated membrane proteins originating at the synaptic vesicle (VAMP), a R/v-SNARE, (Baumert et al., 1989; Trimble et al., 1988) to target proteins for fusion on plasma membrane (Q/t-SNAREs), syntaxin and SNAP-25 (synaptosome associated protein) (Bennett et al., 1992; Oyler et al., 1989; Sogaard et al., 1994). Formation of this complex allows the initiation of the fusion process by serving as a docking site for vesicles (Sogaard et al., 1994). We propose that SNARE proteins are involved in the regulated release of cytotoxic mediators from CTLs and NK cells.

1.2 Results

The t-/Q SNAREs syntaxin-3, syntaxin-4, syntaxin-6, and SNAP-23 mRNA are expressed in CTLs and NK cells

CTLs were subjected to RT-PCR analysis with specific primers, as previously described in chapter 2. CTLs from three different RNA preparations exhibited mRNA expression for syntaxin-3 (462bp), syntaxin-4 (894bp), syntaxin-6 (549bp) and SNAP-23 (493 bp) (Figure 3.1). Similarly, the natural killer cell line, YT-Indy, was also found to express all four t-/Q-SNARE isoforms, syntaxin-3, syntaxin-4, syntaxin-6, and SNAP-23. A positive result was assessed by the presence of PCR product at the expected size compared with appropriate positive control samples. The results showed that CTL and YT-Indy expressed mRNA for syntaxin-3. Syntaxin-3 migrated at 462bp and parallel to the documented control (neutrophil). Syntaxin-4 migrated at 894bp and parallel with the eosinophils control. Similarly, syntaxin-6 migrated to the expected base pair of 549bp and parallel to the documented neutrophil control mRNA. Finally, the expression of SNAP-23 mRNA was documented in both CTL and YT-Indy. SNAP-23 migrated parallel to the eosinophil documented positive control at 493bp. Sequences of the amplified fragments revealed >98% identity with the reported human cDNA sequences for the respective isoforms (sequencing was performed in our lab during previous studies on eosinophils (Logan et al., 2002).

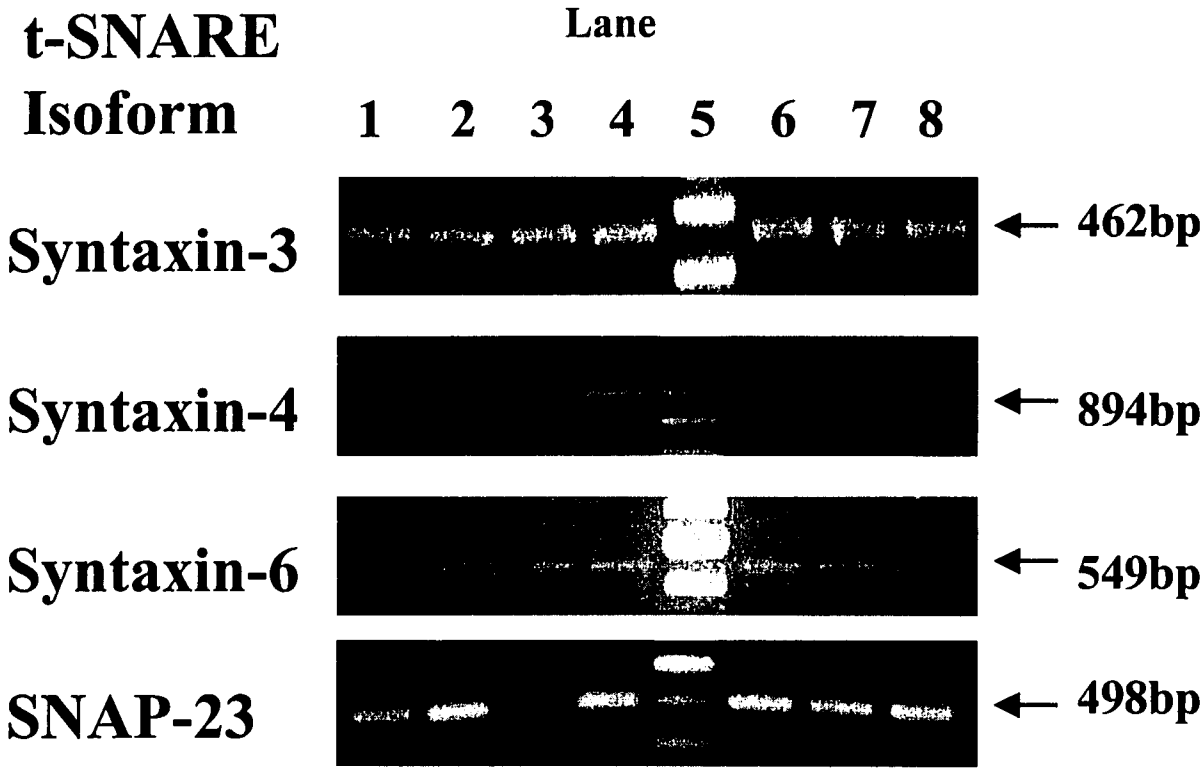

Figure 3.1. Message detection of t-SNARE isoforms in human CD8$^+$T-cell clones and YT-Indy. Using specific primers for t-SNARE molecules, message encoding syntaxin-3(462bp), syntaxin-4(894bp), syntaxin-6 (549bp) and SNAP-23 (498bp) was detected by reverse transcriptase-polymerase chain reaction followed by gel electrophoresis on a 2% agarose gel and staining by ethidium bromide. The housekeeping gene GAPDH was also used (not shown). Documented control cell-types were used as positive controls for each t-SNARE. Lane 1 in each gel represents positive controls for SNARE isoforms: neutrophil cDNA (syntaxin-3, syntaxin-6), eosinophil cDNA (syntaxin-4,SNAP-23). Lane 2-4 represents CD8+ T-cell cDNA. Lane 5 represents the 1000 bp ladder and lane 6-8 represents YT-Indy cell cDNA. One representative PCR product was selected for cloning and sequencing for each. Sequences revealed >95% identity with the reported human cDNA sequences for the respective isoforms.

The v-/R SNAREs, VAMP-1, VAMP-2, VAMP-3, VAMP-7 (TI-VAMP), and VAMP-8, mRNA are expressed in CTLs and NK cells

In order to determine SNARE isoforms expressed in $CD8^+$ CTLs and YT-Indy, specific primers for v-/R SNARE molecules were used to determine the presence of transcripts encoding v-/R SNAREs VAMP-1, VAMP-2, VAMP-7 (TI-VAMP), and VAMP-8 by RT-PCR. The results are shown in Figure 3.2. The results showed that CTL and YT-Indy express mRNA for VAMP-1 at 356bp. VAMP-2 showed similar mRNA expression in CTLs and YT-Indy, with bands present at 348bp and running parallel to the brain control. VAMP-3 showed mRNA expression in all lanes of CTLs and YT-Indy. VAMP-3 migrated at 315bp and parallel with the Caco-2 control cDNA. Similarly, VAMP-7 migrated to the expected base pair of 684bp and parallel to the documented RBL control mRNA. Finally, expression of VAMP-8 mRNA was documented in both CTL and YT-Indy migrating in parallel to the CaCo-2 documented positive control at 587bp. Since both CTLs and NK cells expressed message encoding for the same v-/R and t- /Q SNAREs, this suggested that gene expression was similar in both GrB-containing cells.

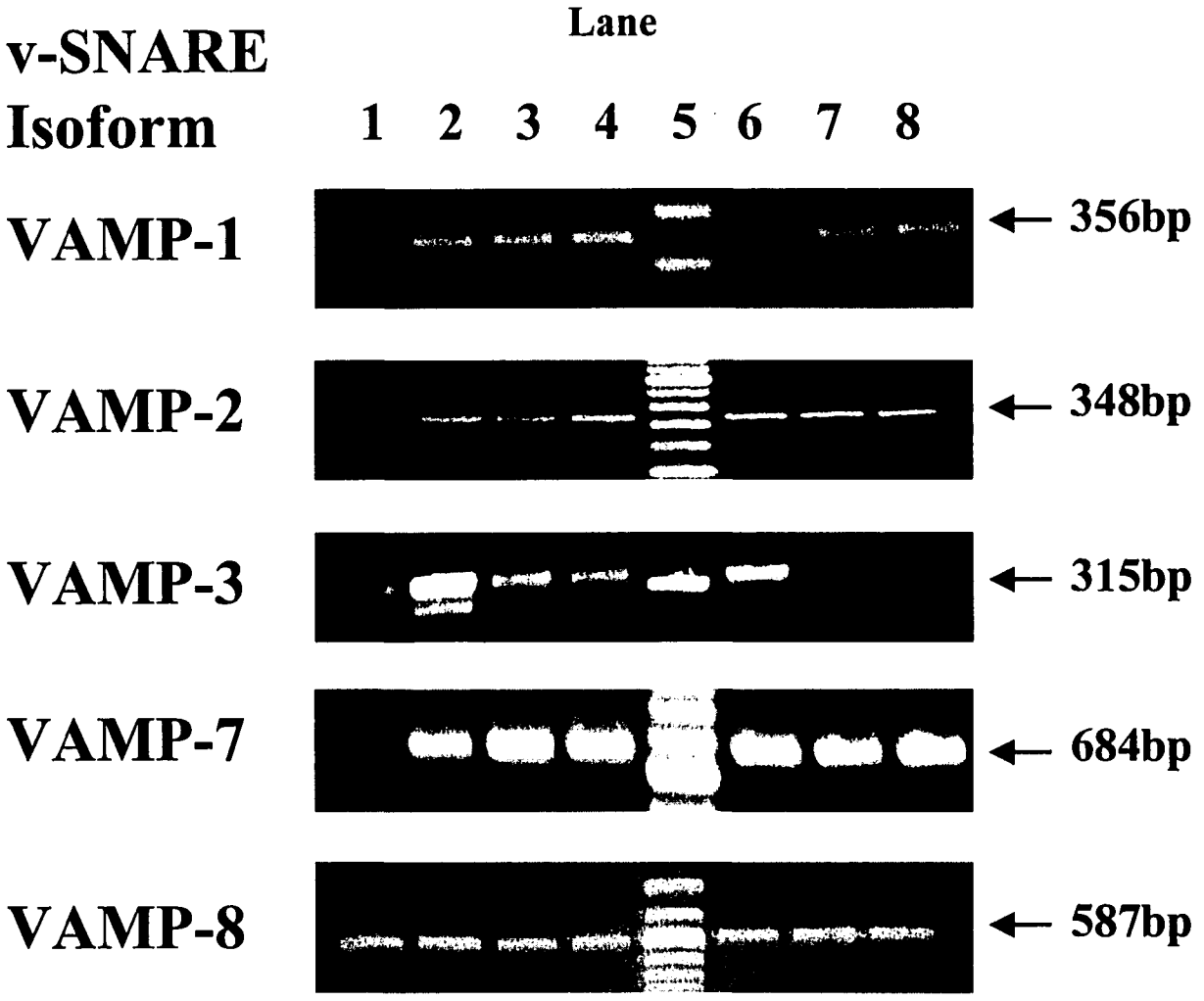

Figure 3.2. Message detection of v-SNARE isoforms in human CD8$^+$T-cell clones and YT-Indy. Using specific primers for v-SNARE molecules, message encoding VAMP-1 (356bp), VAMP-2 (348bp), VAMP-3 (315bp), VAMP-7 (684bp) and VAMP-8 (587bp) was detected by reverse transcriptase-polymerase chain reaction followed by gel electrophoresis on a 2% agarose gel and staining by ethidium bromide. The housekeeping gene GAPDH was also used (not shown). Documented control cell-types were used as positive controls for each v-SNARE. Lane 1 in each gel represents positive controls for SNARE isoforms: human brain cDNA (VAMP-1,VAMP-2), Rat Basophilic Leukemia (RBL) cDNA (VAMP-7), CaCO-2 colonic epithelial cDNA(VAMP-8). Lane 2-4 represents CD8+ T-cell cDNA. Lane 5 represents the 1000 bp ladder and lane 6-8 represents YT-Indy cell cDNA. One representative PCR product was selected for cloning and sequencing for each. Sequences revealed >95% identity with the reported human cDNA sequences for the respective isoforms.

CTLs and NK cells express mRNA for the Rab protein (Rab3d, Rab4a, Rab5a, and Rab27a)

In order to determine the expression of mRNA transcripts of Rab isoforms in $CD8^+$ CTLs and YT-Indy, specific primers and RT-PCR were used. Primer pairs specific for Rab3d, Rab4a, Rab5a, and Rab27a were used. Results of RT-PCR are shown in Figure 3.3. The results showed that CTL and YT-Indy express mRNA for Rab3d, which migrated at 730bp, similar to the positive control (HMC-1). Rab4a showed mRNA expression in all lanes of CTLs and YT-Indy. Rab4a migrated at 622bp and parallel with the positive control, Hela cell cDNA. Similarly, Rab5a migrated at the expected size of 733bp along with products from the neutrophil positive control Similarly, mRNA for Rab27a was expressed in both CTL and YT-Indy. Rab27a migrated to 573bp. GAPDH was control was run for CTL and YT-Indy cDNA samples to confirm the integrity of the cDNA (data not shown).

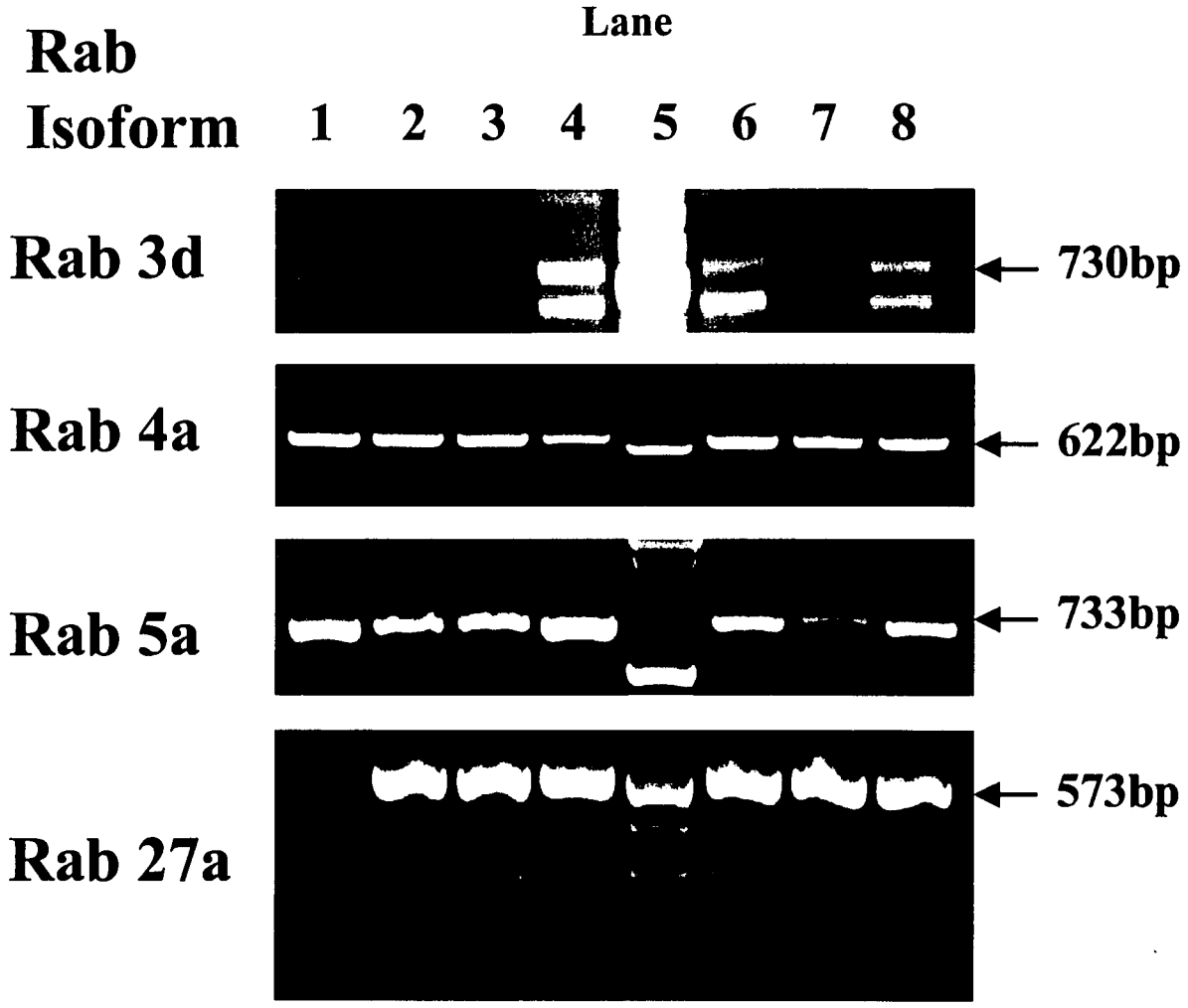

Figure 3.3. Message detection of Rab isoforms in human CD8$^+$T-cell clones and YT-Indy. Using specific primers for Rab molecules, message encoding Rab 3d (730bp), Rab4a (622bp), Rab5a (733bp), and Rab27a(573bp) was detected by reverse transcriptase-polymerase chain reaction followed by gel electrophoresis on a 2% agarose gel and staining by ethidium bromide. The housekeeping gene GAPDH was also used to confirm the presence of the Rab isoform. Lane 1 in each gel represents positive control cDNA Rab3d (HMC-1), Rab4a (Hela), Rab5a(neutrophil), Rab 27a (GAPDH). Lanes 2-4 represent CD8$^+$T-cell clone cDNA, Lane 5 represents a portion of the 100Kbp ladder, lanes 6-8 represent YT-Indy cDNA. Rab isoforms migrated similarly to their expected product size and parallel to the positive controls

CTLs and NK cells express protein for v-/R SNAREs VAMP-2, VAMP-7 (TI-VAMP), VAMP-8, and t-/Q/SNAREs syntaxin-4, syntaxin-6, and SNAP-23

Based on literature and results stemming from out lab, various isoforms of SNAREs were selected to confirm expression of protein. Western blot analysis of cell lysates isolated from CTLs and NK cells confirmed v-/R and t-/Q SNARE isoform protein expression. Using antibodies specific for SNARE proteins, we determined the protein expression of syntaxin-4, syntaxin-6, SNAP-23, VAMP-2, VAMP-7, and VAMP-8 (Figure 3.4). Detection of bands corresponding to the expected molecular weight and running parallel to the appropriate positive control was considered a positive result. Positive controls were as follows: syntaxin-4, SNAP-23 (platelet cell lysate) (Chen et al., 2000; Feng et al., 2002), syntaxin-6 (neutrophil cell lysate) (Sesack and Snyder, 1995), VAMP-7 (rat brain homogenates) (Advani et al., 1999; Sesack and Snyder, 1995), VAMP-8 (platelet cell lysate) (Polgar et al., 2002). In both CTLs and YT-Indy, syntaxin-4 produced a band at the expected 35kDa and ran parallel to the documented platelet lysate control. Similarly, in YT-Indy and CTLs, SNAP-23 migrated correspondingly to platelet lysate positive control and at the expected molecular weight of 23kDa. Syntaxin-6 showed much weaker protein expression in both CTLs and NK cells, compared to syntaxin-4 and SNAP-23. There appeared to be only weak bands at the expected molecular weight of 30KDa in both CTL and YT-Indy lysates. A strong positive control band of rat brain homogenate at the correct molecular weight was observed. The same protein concentration was loaded for CTL and YT-Indy lysates and rat brain homogenate. CTL and YT-Indy cell lysates were examined for confirmation of protein expression of

v-/R SNARE isoforms. VAMP-7 showed protein expression in both CTLs and NK cells. Both CTL and NK cell lysates migrated to the expected molecular weight of 20KDa and corresponded to the documented positive control of rat brain homogenate. When the v-/R SNARE VAMP-8 was examined there was expression of VAMP-8 in CTL lysates. Band migrated to 15KDa and parallel to platelet lysate positive controls. However, it appeared that there were no detectable levels of VAMP-8 expression in YT-Indy cell lysates. Since a protein product was not observed for VAMP-8 in YT-Indy cell lysates, but observed in CTL lysates further analysis using confocal microscopy was performed to determine localization of VAMP-8.

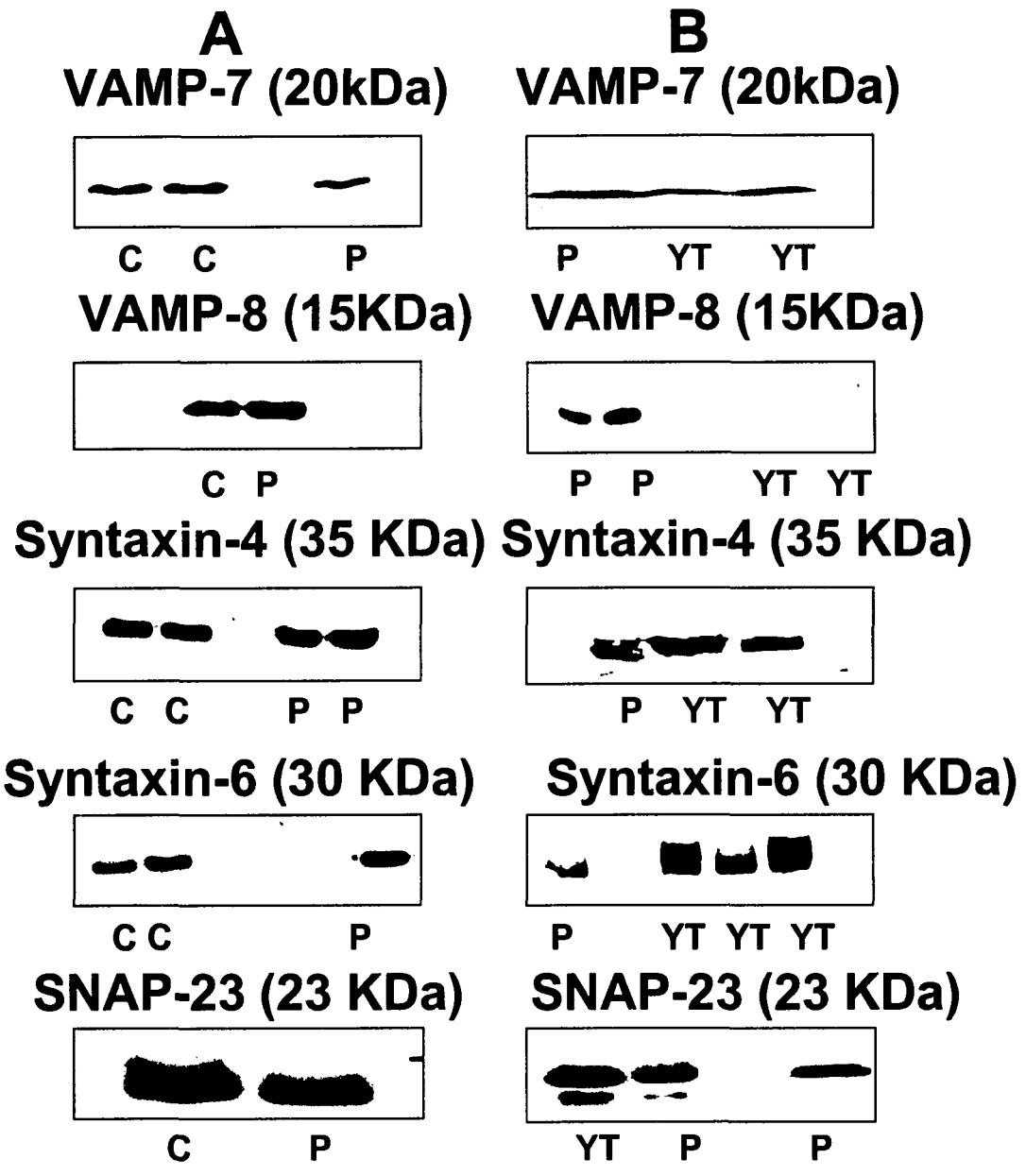

Figure 3.4 Western blot analysis of SNARE isoforms in human CD8$^+$ T-cell clones and YT-Indy. **A)** Protein expression of SNARE isoforms in CTLs. V-SNAREs VAMP-7 and VAMP-8 had protein expression. VAMP-7 migrated at 20KDa and accordingly to its documented positive control, platelet lysate. Similarly, VAMP-8 migrated to the expected molecular weight of 15KDa and parallel to a platelet lysate control. Protein expression for the t-SNAREs syntaxin-4, syntaxin-6 and SNAP-23 were examined in CTLs. Syntaxin-4 and syntaxin-6 migrated at 35KDa and 30KDa, respectively and both ran parallel to platlet lysate control. SNAP-23 migrated at 23KDa in CTLs as expected and parallel to platelet positive control. **B)** YT-Indy were examined for protein expression of the same SNARE isoforms as CTLs. All t-SNAREs migrated parallel to their positive controls and at the expected molecular weight. The v-SNARE isoforms, VAMP-7 showed expression of protein in YT-Indy cell lysates, however, no expression of VAMP-8 was detected in lysates.

C-CTL lysate; YT-YT-Indy lysate; P-platelet lysate

Identification of Granzyme-B and SNARE localization in CTLs and YT-Indy by Confocal Microscopy

Confocal analysis confirmed the presence of SNARE isoforms in CTLs and NK cells as well as determined the localization of the proteins. In addition, colocalization studies helped to speculate further if SNAREs are in fact involved in exocytosis of GrB from granules. Table 4 shows a detailed description of the slide preparations for CTL and YT-Indy immunocytochemistry.

We examined intracellular staining in human CTL clones and YT-Indy with specific R-SNARE antibodies (VAMP-7, VAMP-8) and with the granule markers, CD63 and GrB. Shown are representative images. In all cases, irrelevant mouse IgG_1 (or rabbit serum) was used as a negative control. The negative controls were stained with secondary antibodies similarly to experimental slides; however, mouse IgG_1 (or rabbit serum) replaced the specific SNARE antibody. Isotypes control images and experimental images were taken under the same settings and showed little or no immunoreactivity to any of the investigated antibodies. Cell integrity was confirmed by using DIC (Differential Interference Contrast).

Localization of VAMP-7 (TI-VAMP) and GrB in CTLs and YT-Indy

Cells were stained with Alexa 594 goat anti-mouse secondary antibody (red) to detect immunoreactivity against goat anti-mouse VAMP-7 and stained with streptavadin-FITC (green) to detect immunoreactivity against GrB. In YT-Indy a significant portion of cytoplasm expressed both GrB and VAMP-7, as expected (Figure 3.5). However, the amount of colocalization, indicated by yellow fluorescence, was minimal, with only a few areas showing a yellow color indicated by the arrows. In CTLs, VAMP-7 is

distributed to the periphery of the cell with a localized population to one end of the cell. GrB has a similar distribution with more punctate staining, which may be an indication of granule populations. Despite localization to the same region of the cell, VAMP-7 and GrB only appear to have minimal colocalization in stimulated CTLs.

Localization of VAMP-8 and GrB in CTLs and YT-Indy

Cells were stained using a donkey anti-rabbit Rhodamine-Red-X-conjugate secondary antibody (red) to detect immunoreactivity against VAMP-8. Staining with streptavadin-FITC (green) was used to detect immunoreactivity against GrB. In YT-Indy, GrB was mainly localized to the cytoplasmic portion of the cell, the expected location of GrB (Figure 3.6). However, when looking at the staining pattern of VAMP-8 it seemed that VAMP-8 was localized to the nucleus rather than cytoplasm of YT-Indy cell. There was no detectable colocalization between GrB and VAMP-8. Therefore, VAMP-8 may play a minimal role, if any, in GrB release. Although the localization of VAMP-8 to the nucleus of YT-Indy is intriguing, this may just be an artifactual finding unless confirmed using other methods. In CTLs, GrB was localized to the cytoplasm, while VAMP-8 also had a cytoplasmic distribution. Regardless of this similar individual staining pattern, when the two are merged the amount of colocalization appears to be minimal at best.

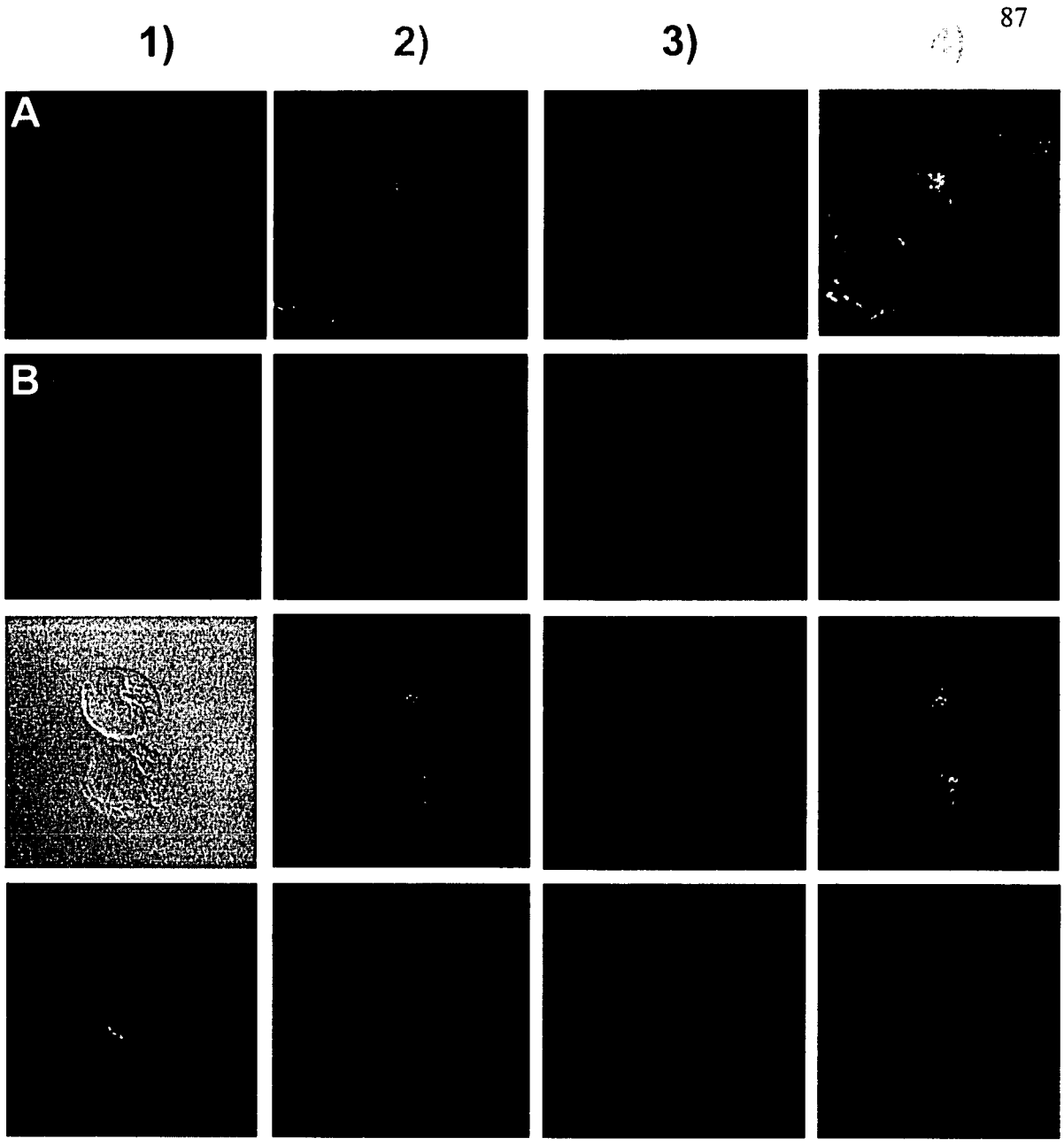

Figure 3.5 Confocal microscopic analysis of GrB and VAMP-7 in YT-Indy and CTLs.

Column 1 represents either the DIC (differential interference contrast) image or DAPI (nuclear stain, blue), column 2 represents GranzymeB staining (green), column 3 represents VAMP-7 staining (red), column 4 represents the merged image (yellow). Row A represents YT-Indy, row B is YT-Indy isotype control, row C represents stimulated CTLs, row D represents stimulated CTL isotype control.

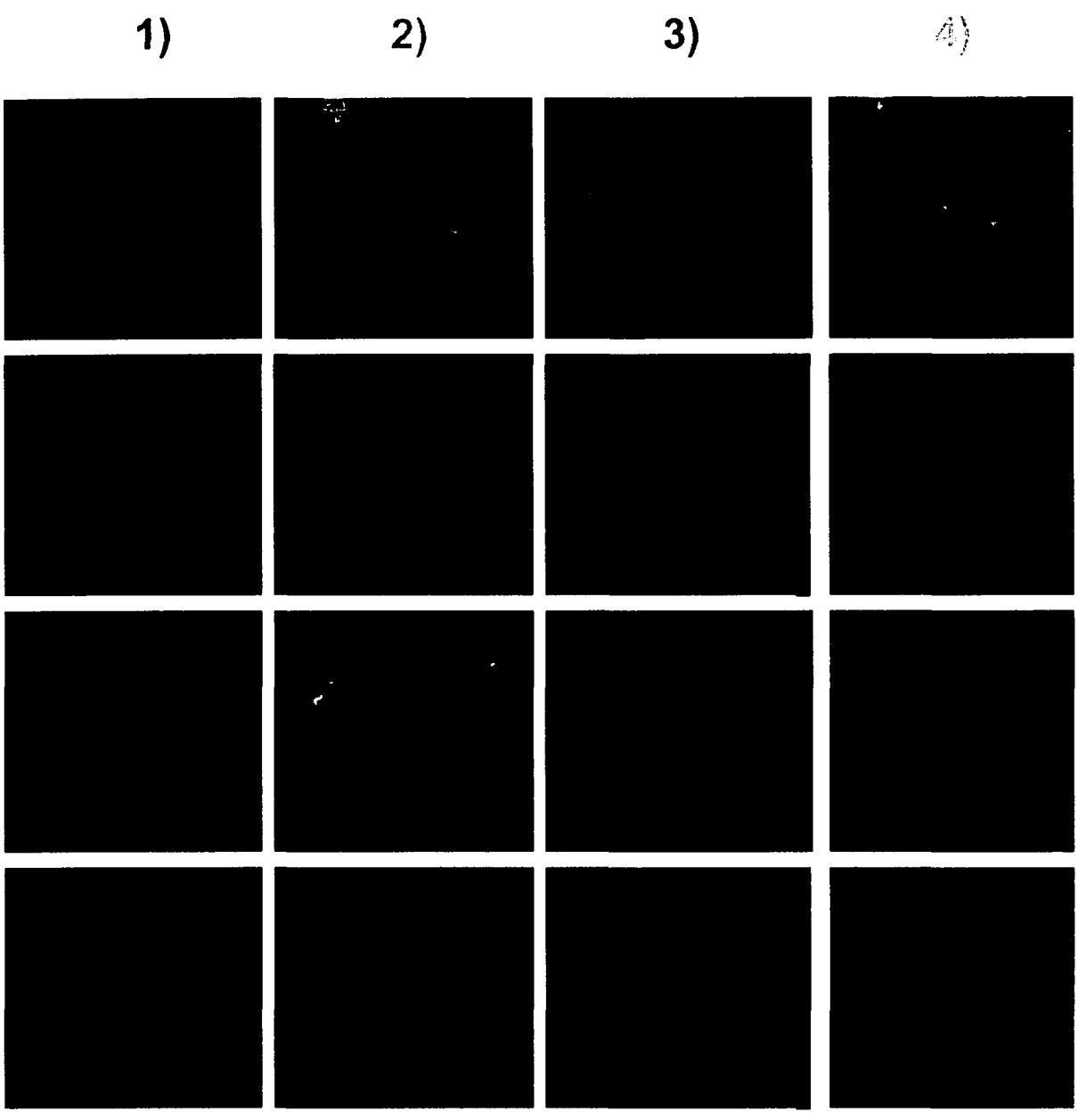

Figure 3.6 Confocal microscopic analysis of GrB and VAMP-8 in YT-Indy and CTLs.

Column 1 represents or DAPI (nuclear stain, blue), column 2 represents GranzymeB staining (green), column 3 represents VAMP-8 staining (red), column 4 represents the merged image (yellow). Row A represents YT-Indy, row B is YT-Indy isotype control, row C represents stimulated CTLs, row D represents stimulated CTL isotype control.

Localization of VAMP-7 (TI-VAMP) and CD63 in CTLs and YT-Indy

Cells were stained with Alexa 594 goat anti-mouse secondary antibody (red) to detect immunoreactivity against VAMP-7 and stained with BODIPY-FL (green) to detect immunoreactivity against CD63. In YT-Indy (Figure 3.7), CD63 was primarily localized to the periphery of the cell, as this is the expected localization of CD63 as it is found in lysosomes and SL. VAMP-7 exhibited a cytoplasmic distribution. When the two images were merged, in YT-Indy a significant portion of cytoplasm expressed both CD63 and VAMP-7, indicating the possible association of VAMP-7 with SL. This was evident by the merging of red (VAMP-7) and green (CD63) to generate yellow fluorescence. In CTLs, both CD63 and VAMP-7 are localized to the cytoplasmic portions of the cell. However, in comparison to YT-Indy, stimulated CTLs display much less colocalization as evidence by the reduced amount of yellow fluorescence.

Localization of VAMP-8 and CD63 in CTLs and YT-Indy

Cells were also stained using a donkey anti-rabbit Rhodamine-Red-X-conjugate secondary antibody (red) to detect immunoreactivity against VAMP-8 and stained with BODIPY-FL (green) to detect immunoreactivity against CD63. In YT-Indy, CD63 is found localized to the peripheral region of the cell, however, unexpectedly VAMP-8 appeared to be completely localized to the nuclear region of the cell rather than the cytoplasmic region as would be expected from literature (Hong, 2005). The role of VAMP-8 in the nucleus, if any, is unclear. VAMP-8 may be involved with trafficking from the nucleus to the cytoplasm or perhaps this phenomenon is cell line specific. Further investigation of human NK cells would be needed to confirm VAMP-8

specificity to the nucleus. From these results, it appeared that VAMP-8 and CD63 do not have a common role in YT-Indy cells. In stimulated CTLs, CD63 was primarily localized to the cytoplasm and VAMP-8 showed cytoplasmic distribution. It appears in stimulated CTLs that a significant portion of cytoplasm expressed both CD63 and VAMP-8, indicating the possible association of VAMP-8 with SL. This was evident by the merging of red (VAMP-7) and green (CD63) to generate yellow fluorescence. In unstimulated CTLs, VAMP-8 does appear to be present around the cytoplasm of the cell, while CD63 only appears to be present in minimal amounts and localized to the cytoplasmic region. A possible explanation may be that $CD63^{+}$ granules only appear after stimulation of CTLs by a target cell.

Nonetheless, these results should be interpreted with caution due to the limitations of confocal microscopy. While confocal microscopy is a valuable tool, in the absence of sophisticated software capable of detecting the colocalization of two molecules, should only provide confirmatory support rather than form the basis of a conclusion. Confocal microscopy may offer a resolution that is not better than 0.1 microns. This may lead to misinterpretation of data, especially in the case of double labeling, with a view to determining whether two structures are or are not distinct.

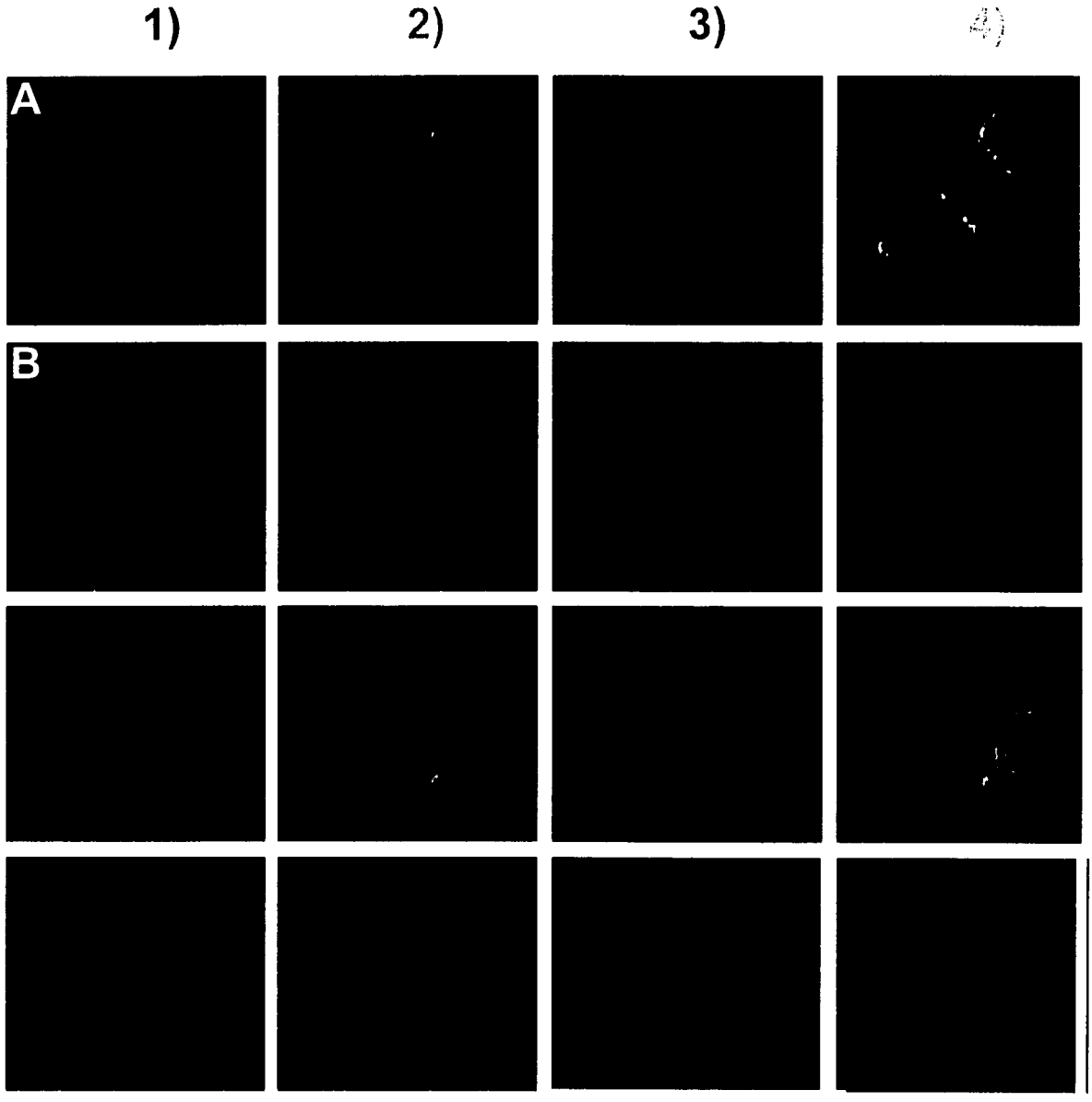

Figure 3.7 Confocal microscopic analysis of CD63 and VAMP-7 in YT-Indy and CTLs.

Column 1 represents DAPI (nuclear stain, blue), column 2 represents CD63 staining (green), column 3 represents VAMP-7 staining (red), column 4 represents the merged image (yellow). Row A represents YT-Indy, row B is YT-Indy isotype control, row C represents stimulated CTLs, row D represents stimulated CTL isotype control, row E represents unstimulated CTLs.

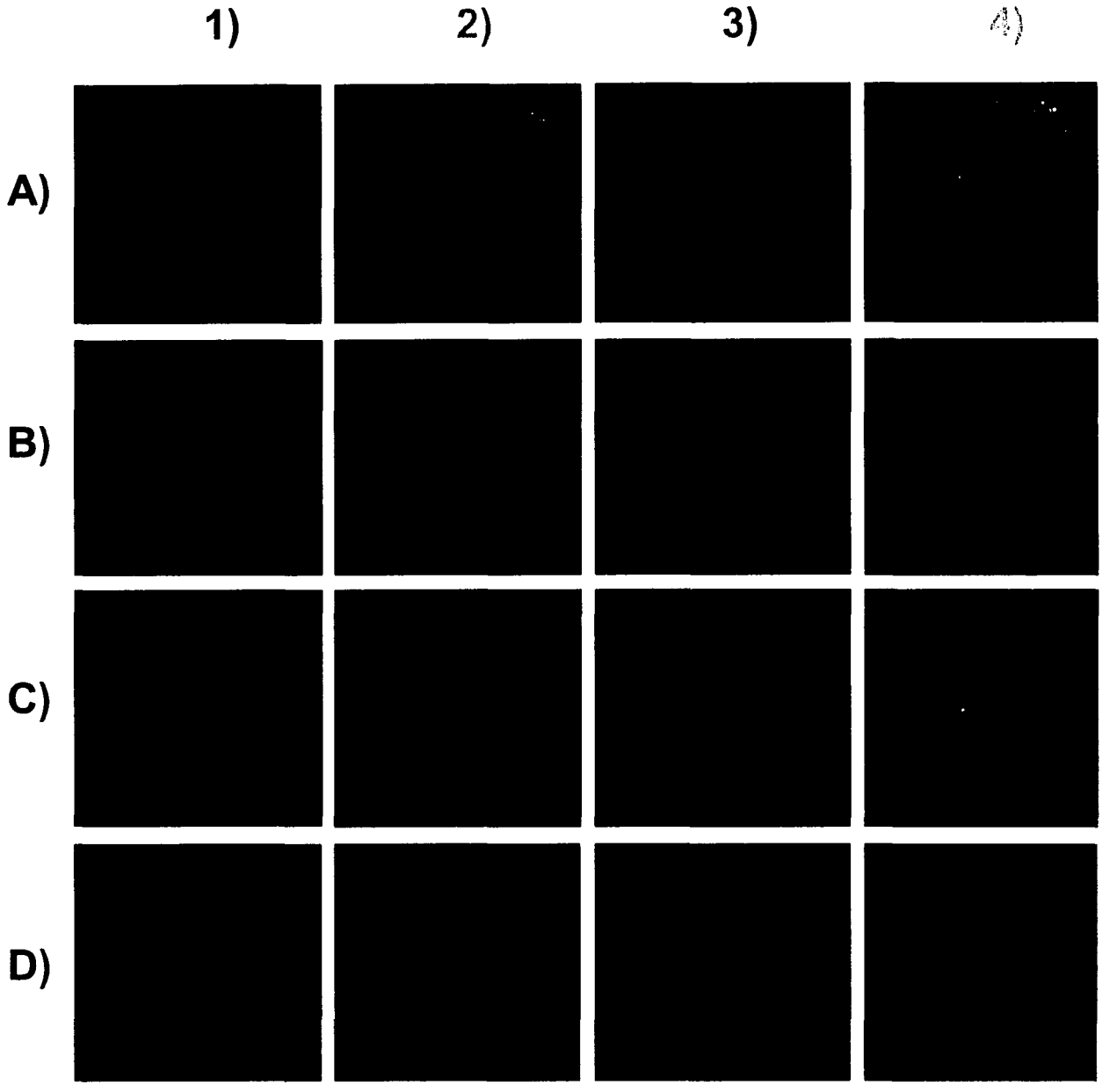

Figure 3.8 Confocal microscopic analysis of CD63 and VAMP-8 in YT-Indy and CTLs. Column 1 represents DAPI (nuclear stain, blue), column 2 represents CD63 staining (green), column 3 represents VAMP-8 staining (red), column 4 represents the merged image (yellow). Row A represents YT-Indy, row B is YT-Indy isotype control, row C represents stimulated CTLs, row D represents stimulated CTL isotype control.

Table 4- Slide Preparation Layout For Confocal Microscopy

Slide	primary	secondary	Second Primary	Second Secondary	F'(ab)
Exp 1	VAMP-7	Alexa 594			No
Iso 1	mIgG1	Alexa 594			No
Exp2	VAMP-8	rRR			No
Iso2	rIgG1	rRR			No
Exp3	GrB:biotin	Strept-FITC			No
Iso3	mIgG:biotin	Strept-FITC			No
Exp4	CD63	mRR			No
Iso 4	mIgG1	mRR			No
Exp 5	VAMP-7	Alexa 594	GrB:biotin	Strept-FITC	No
Iso 5	mIgG1	Alexa	mIgG:biotin	Strept-FITC	No
Exp 6	VAMP-8	rRR	GrB:biotin	Strept-FITC	No
Iso 6	rIgG1	rRR	GrB: biotin	Strept-FITC	No
Exp 7	VAMP-7	Alexa594	CD63	BODIPY-FL	Yes
Iso 7	mIgG1	Alexa594	mIgG1	BODIPY-FL	Yes
Exp8	VAMP-8	rRR	CD63	BODIPY-FL	No
Iso 8	rIgG1	rRR	mIgG1	BODIPY-FL	No
Exp9	CD63	mRR	GrB:biotin	Strept-FITC	No
Iso 9	mIgG1	mRR	mIgG1:biotin	Strept-FITC	No

Exp: Experimental; Iso: isotype control; mIgG1: mouse immunoglobulin1; rIgG1: rabbit immunoglobulin1; rRR: Rhodamine-Red-X-conjugated affinity purified donkey anti-rabbit IgG; mRR: Rhodamine Red-X-conjugated affinity purified goat anti-mouse IgG; GrB:biotin: granzymeB biotinylated; Strept-FITC: streptavadin conjugated FITC

Discussion

This study has demonstrated that CTLs and NK cells express various isoforms of SNARE molecules. The presence of SNAREs in both cell populations, suggests that SNARE-dependent exocytosis may exist. Since little work has been done concerning SNAREs in CTLs and NK cells, our findings are the first to suggest the possible involvement of SNAREs in the regulated release of GrB from CTLs and NK cells. Similar SNARE profiles have been found in other haematopoietic cells (Shukla et al., 2001), suggesting that SNAREs may be lineage specific.

CTLs and NK cells express message for a number of SNARE isoforms including VAMP-1, VAMP-2, VAMP-3, VAMP-7, VAMP-8, syntaxin-3, syntxin-4, syntaxin-6, and SNAP-23. Based on available literature (Shukla et al., 2001), protein expression of only selected SNAREs was confirmed. Thus, protein expression was determined for v-/R SNAREs VAMP-7, and VAMP-8 and t-/Q SNAREs syntaxin-4, syntaxin-6 and SNAP-23.

The t-/Q-SNAREs, SNAP-23, syntaxin-4, and syntaxin-6 were expressed in NK cells and CTLs by RT-PCR and confirmed by Western blot analysis and, therefore, may be potential docking partners for R-SNAREs in forming the docking complex during exocytosis of SLs.

Upon examination of v-/R SNAREs, VAMP-7 (TI-VAMP) expression was confirmed in both CTL and NK cell lysates. Recent work (Logan et al., 2003) in eosinophils and neutrophils suggested that VAMP-7 may in fact be the most important v-SNARE involved in exocytosis as inhibition of the release of granule contents was observed when antibodies against VAMP-7 were introduced into the cell by streptolysin-

O permeabilization (Logan et al., 2003). Additionally, Martinez-Arca et al., (2003) have suggested that tetanus-insensitive SNARE isoforms (VAMP-7), rather than the tetanus toxin sensitive VAMP-2, may be the most critical R-SNARE for release of stored products in other cell types. However, the exact role of VAMP-7 in CTL and NK granule exocytosis remains unknown.

Immunocytochemistry and confocal microscopy helped to detect the localization of VAMP-7 to determine if VAMP-7 could be a putative partner involved in exocytosis of CTL and NK SLs. When examining the relationship of VAMP-7 with the secretory granule marker, CD63, it appears that in YT-Indy there was strong colocalization of CD63 and VAMP-7. This result suggests that VAMP-7 may be associated with the SL and lysosomal marker CD63. Thus, VAMP-7 may be involved in release of contents from this population. When YT-Indy was examined for colocalization of VAMP-7 and GrB, it appeared that there were significant amounts of expression of GrB and VAMP-7 in the cytoplasmic region of the cell; however, the amount of colocalization between the molecules was minimal. These results, taken collectively, suggest that VAMP-7 is associated with $CD63^+$ granules. However, VAMP-7 is not associated with GrB^+ granules in YT-Indy. This leads to the interesting notion of two distinct granule populations, SL and lysosomes, with VAMP-7 being associated with lysosomes rather than SL. It is interesting to note that cells such as platelets and melanosomes have been shown to express both types of granule populations (Raposo et al., 2001). Therefore, the possibility that NK cells also express these two populations cannot be ruled out.

When I examined stimulated CTLs for expression of VAMP-7 and its association with SL components GrB and CD63, it appeared that stimulated CTLs expressed VAMP-

7, CD63, and GrB in the cytoplasmic region of the cell. However, the amount of colocalization between them was minimal. CD63 and VAMP-7 did not show substantial amounts of colocalization nor did VAMP-7 and GrB. These results suggested that VAMP-7 might not be associated with SL populations in stimulated CTLs, as VAMP-7 did not show significant colocalization with either of the granule markers, CD63 and GrB. It may be that only small portions of SLs express VAMP-7 at distinct time points during maturation. Although there was not complete localization of GrB and VAMP-7 (TI-VAMP) in either cell type, the expression of TI-VAMP may be short lived and be only expressed on granules in distinct maturation times or may be rapidly recycled into the granule.

Immunocytochemistry and confocal microscopy helped to determine the localization of VAMP-8 to determine if VAMP-8 could be a putative partner of t-/Q SNARES in exocytosis of CTL and NK SLs. The surprising finding was that in YT-Indy, VAMP-8 was not associated with the secretory granule marker, CD63 (reviewed in Hong, 2005), but unexpectedly appeared to be localized to the nucleus of the cell. Since I did not have the time to address this observation carefully by immunoprecipitation of nuclear protein with VAMP-8 and to follow that by cloning and sequencing the VAMP-8 band to determine the identity of this protein, I have to assume that this is a controversial finding requiring further careful investigation. GrB localization was also to the cytoplasmic portion of the cell. Therefore, from these observations it appears that VAMP-8 may not be associated with the SL population found in NK cells as VAMP-8 is nuclear and CD63 and GrB are cytoplasmic. This study is the first to report such a unique finding. Additionally, relying solely on confocal microscopy for this finding is limited.

It is, however, important to bear in mind that the YT-Indy cell line comes from a patient with acute lymphoblastic lymphoma and therefore the cell line may lack post-translational modifications necessary for transport of VAMP-8 to the SL or to the cytoplasm of the cell. As indicated, further investigation of human NK cells are needed to confirm VAMP-8 specificity to the nucleus, however, the localization of VAMP-8 to the nucleus agrees with the Western blot results. Previous studies (Antonin et al., 2000; Steegmaier et al., 2000) described VAMP-8 to be localized to early endosomes and the *trans*-Golgi, in other cells. It is interesting that cell lysates did not show VAMP-8 protein expression.

When examining CTLs for VAMP-8 expression contrary to YT-Indy, the distribution of VAMP-8 in CTLs was cytoplasmic. Granule markers CD63 and GrB displayed a cytoplasmic distribution. CTLs appear to show some degree of colocalization between VAMP-8 and CD63. However, VAMP-8 and GrB do not seem to colocalize. This suggests that GrB and VAMP-8 may not be related and the staining pattern observed may be due to VAMP-8 localization to the endosomes or the ER. This may also indicate a localization of VAMP-8 to lysosomes rather than SL, since GrB and VAMP-8 were not colocalized.

We report for the first time that CTLs and NK cells express the t-SNAREs, SNAP-23, and syntaxin-4, which may play an important role in docking and exocytosis of CTL and NK cell vesicular contents. Both CTLs and NK cells express mRNA for syntaxin-4 and SNAP-23. In addition, both showed abundant protein expression by Western blot analysis for the t-/Q SNAREs. These results agree with previous studies (Paumet et al., 2000; Ravichandran et al., 1996) stating that syntaxin-4 and SNAP-23 are

important in the formation of the SNARE complex in RBL mast cells and non-neuronal tissues.

Message for syntaxin-6 was expressed in both CTLs and YT-Indy. Syntaxin-6 also expressed protein in both cell types, however, the abundance of syntaxin-6 expression seemed to be much less than the amount of protein detected for other v-/Q SNAREs, including syntaxin-4 and SNAP-23 at the same concentration of protein loaded. Other studies (Wendler et al., 2001) have shown syntaxin-6 to be localized to secretory granules. Although this is not ruled out, it seems highly unlikely that syntaxin-6 is a major t-/Q SNARE involved in exocytosis of granules from CTLs and NK cells.

While confocal microscopy is a valuable tool, limitations of the technique do exist and conclusions should not be solely based on confocal microscopy (Brelje et al., 2002). The first limitation of confocal microscopy is the resolution, which is limited by the wavelength of light. Although the resolution limits of the microscope are pushed, the resolution is not better than 0.1 microns under ideal settings.

Further studies are required to determine the exact role of SNAREs in exocytotic events of CTL and NK cell granule populations. Specific proteins may be involved which may modulate the secretory function of these important cytotoxic lymphocytes.

Acknowledgements

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Chapter 4

General Discussion

I. Summary of SNAREs and Rabs in CTLs and NK Cells

The observations arising from this research project are novel and interesting. I have described the expression of SNARE and Rab proteins in human $CD8^+$ T-cell clones and an NK cell line, YT-Indy using RT-PCR, Western blot analysis, immunocytochemistry and confocal microscopy. It still remains unclear, however, what the precise functional role of SNARE isoforms may be in the exocytosis of stored mediators from CTL and NK SLs. The Q-SNAREs, SNAP-23, syntaxin-4, and syntaxin-6 were expressed in NK cells and CTLs and may be potential docking partners for R-SNAREs during exocytosis. R-SNAREs TI-VAMP, and VAMP-8 were differentially expressed by confocal microscopy and Western blot analysis. TI-VAMP message was expressed in both CTLs and YT-Indy. To confirm the presence of TI-VAMP, I carried out Western blot analysis and confirmed that this protein was expressed in both CTLs and YT-Indy. This suggests that TI-VAMP may in fact be the most important R-SNARE involved in granule exocytosis. This finding would agree with recent results by Logan et al. 2005 (submitted) in human neutrophils and eosinophils. The data presented thus far, suggest a possible association of VAMP-7 (TI-VAMP) involvement in secretion of GrB from YT-Indy SL. However, the study remains incomplete without functional data. Confocal microscopy showed a punctate staining of

TI-VAMP with partial colocalization with GrB. This may also suggest the presence of TI-VAMP in other vesicles besides those that are GrB^+. TI-VAMP also showed colocalization with CD63, which suggests a possible role of TI-VAMP in the release of $CD63^+$ granules including GrB release. Although there was no complete localization of GrB and TI-VAMP in either cell type, the expression of TI-VAMP may be short lived and be only expressed on granules at distinct maturation times.

VAMP-8 was shown to express message in both CTL and YT-Indy, although detectable protein levels were found only in CTL lysates. YT-Indy lysates did not seem to express detectable levels of VAMP-8 protein. Confocal analysis verified my Western blot data. Confocal microscopy also showed an unexpected perinuclear VAMP-8 staining in CTLs, with minimal colocalization with GrB. The staining observed for VAMP-8 in CTLs may suggest localization to the ER or transport vesicles. In addition, VAMP-8 appeared to be localized to the nucleus of YT-Indy cells only (and not CTLs) and therefore, may not play a role in exocytosis of SLs. Confocal images, and perhaps data from Western blot analysis showing that VAMP-8 immunoreactivity was absent in the post-nuclear supernatants of YT-Indy cells, may provide some incomplete evidence for a possible association between this v-SNARE and the nucleus in YT-Indy cells. .The function of VAMP-8 in the nucleus can only be speculated. Previous studies (Antonin et al., 2000; Steegmaier et al., 2000) have found VAMP-8 localized to early endosomes and the trans Golgi, in other cells, suggesting the role of VAMP-8 to be cell specific. Again, it is important to emphasize the need to follow this observation with further studies including cloning and sequencing the VAMP-8 band obtained following immunoprecipitation of nuclear proteins with VAMP-8 to verify the identity of the

protein. This may be an important observation that merits further investigation in both human NK cells and CTLs and to address the issue of whether the observation is valid or an artifact

In this project I reported that CTLs and NK cells express the t-SNAREs, SNAP-23 and syntaxin-4, which may be important in docking and exocytosis of CTL and NK cell granules. These results agree with previous studies (Paumet et al., 2000; Ravichandran et al., 1996) stating that syntaxin-4 and SNAP-23 are important in the formation of the SNARE complex in RBL mast cells and non-neuronal tissues. These alternative t-SNAREs have been described in a variety of non-neuronal cell types (Dvorak et al., 1994; Lacy et al., 2001; Martinez-Arca et al., 2001; Shenoy et al., 2003; Wardlaw et al., 1995). In support of previous finding in our lab (Logan et al., 2002; Logan et al., 2003) it appears that SNARE proteins may be important components of the machinery involved in SL secretion from CTLs and NK cells. Although further studies are needed, it seems the t/Q- SNAREs SNAP-23 and syntaxin-4 most likely are the docking partners to a yet to be determined VAMP protein. The presence of SNAREs in both cell population, suggests that SNARE-dependent exocytosis may be important in granule exocytosis. Since little work has been done concerning SNAREs and CTLs and NK cells, my findings are the first to suggest the involvement of SNAREs in the regulated release of GrB from CTLs and NK cells

Although previous work on neutrophils has shown syntaxin-6 to be an important t-SNARE involved in docking and fusion of neutrophil granules, (Martin-Martin et al., 2000), the results obtained in Western blot analysis in this study suggest that syntaxin-4 is predominant. Other studies (Wendler et al., 2001) have also shown syntaxin-6 to be

localized to secretory granules. Although this is not ruled out, it seems highly unlikely that syntaxin-6 is the major t-/Q SNARE involved in exocytosis of granules from CTLs and NK cells.

Although much is known about the steps leading to exocytosis of cytotoxic granule contents, the precise mechanisms involved in the critical process of docking and fusion of GrB-containing granules or vesicles remains unclear. It is likely that the v/R-SNAREs identified in this study play specific roles in the docking and fusion step; the same may be true for the t/Q-SNAREs. Although a minimum of three proteins (one v-SNARE and two t-SNAREs) are needed to form the core of the fusion complex, it remains unclear which specific are required in CTLs and NK cells. It is possible that different SNARE proteins may only play a role in specific stages of the cell maturation cycle, or that some may only be present for milliseconds before getting recycled back into the cytoplasm. Further studies are required to determine the exact role of SNAREs in exocytotic events of CTL and NK cell granule populations. Specific proteins may be involved which may modulate the secretory function of these important cytotoxic lymphocytes.

Rabs mRNA was expressed in both CTLs and NK cell suggesting a role for Rabs in upstream events, which may regulate the docking process. More studies on Rabs are needed to determine the precise Rabs proteins and their role in exocytosis of granules from CTLs and NK cells.

II. Regulatory Proteins of Exocytosis: A Closer Look

v-/R-SNARES

This study is the first to report the localization of VAMP-8 to the nucleus of YT-Indy cells, although this remains contraversial and requires further careful elucidation. It also appears that VAMP-8 may not be associated with $CD63^+$ compartments nor GrB^+ compartment, suggesting that VAMP-8 may have a negligible role to play in the exocytotic release from SL in YT-Indy. Since there was no interaction between VAMP-8 and GrB in CTLs, it seems likely that VAMP-8 may not be involved in GrB release from SL. VAMP-8 and CD63 marginally colocalized and purposes that VAMP-8 may not play a role in secretion from SL in either CTLs or YT-Indy NK cells.

The majority of literature localized VAMP-8 to the early endosomes and compartments associated with the endosomes such as TGN, late endosomes and plasma membrane (Antonin et al., 2000; Mullock et al., 2000; Nagamatsu et al., 2001; Steegmaier et al., 2000; Wong et al., 1998). Therefore, as we can see the exact localization of SNAREs is difficult to determine and it may be in fact that SNAREs function differently in different cell types.

VAMP-7 (TI-VAMP) has mainly been localized to neuronal cell types (Galli et al., 1998), however recent work in eosinophils and neutrophils (Logan et al., 2005, submitted) has suggested the role of VAMP-7 in mediating exocytosis from granules. This study has shown that VAMP-7 (TI-VAMP) is localized to $CD63^+$ granules in YT-Indy, and therefore, may be involved in exocytosis of SL. VAMP-7, however, showed only minimal colocalization with GrB. Therefore, it is possible that VAMP-7 may be located on lysosomes rather than SL in YT-Indy. That in CTLs, only minimal

colocalization was found between VAMP-7 and CD63 or GrB, suggest that perhaps only a small portion of SL express VAMP-7 depending on granule maturation. Plasma membrane-enriched fractions may help to determine the exact localization and the role of VAMP-7 in CTLs and NK cells. These results would agree with data from Coco et. al., 1999, which showed that VAMP-7 (TI-VAMP) is colocalized to $CD63^{+}$ compartments in neurons (Coco et al., 1999). Additionally, VAMP-7 (TI-VAMP) was also found to localize to late endosomes and lysosomes in other non- neuronal cells, further supporting this studies findings (Advani et al., 1999; Martinez-Arca et al., 2003; Rao et al., 2004; Ward et al., 2000). VAMP-7 (TI-VAMP) has been shown to be present in rat tissue (Advani et al., 1999) and localized to various cell type granules including CD63+ granules (Advani et al., 1999; Martinez-Arca et al., 2003; Rao et al., 2004; Ward et al., 2000). Additionally, VAMP-7 (TI-VAMP) has been shown to be localized to the trans-Golgi network, and to transport vesicles (Advani et al., 1999; Galli et al., 1998). VAMP-7 (TI-VAMP) has been suggested by (Ward et al., 2000) to be involved in late endosome and lysosome fusion events as the introduction of neutralizing antibodies to VAMP-7 inhibited homotypic fusion events of lysosomes, but not of early endosomes. The role of VAMP-8 is still unclear as there are contrasting studies by Antonin et al., 2000 and Mullock et al. 2000 in determining inhibitory effects of anti-VAMP-8 antibodies in endosome, late endosome and lysosome fusion. Taken together, including my data, it seems that the role of VAMP-8 remains unclear requiring focused attention in order to pin-point its precise function in these cells.

While VAMP-2 has been expressed in other haematopoietic cells such as platelets (Feng et al., 2002; Logan et al., 2003), mast cells (Paumet et al., 2000), epithelial cells

(Proux-Gillardeaux et al., 2005; Weimbs et al., 2003), and neutrophils (Brumell et al., 1995), my studies did not show detectable immunoreactivity in either CTL or NK cell lysates. This agrees with previous results from our lab on work with eosinophils (Lacy et al., 1999). Preparations of granule-enriched fractions are needed to concentrate the VAMP-2 protein to detectable limits.

t-/Q SNAREs

SNAP-23 and syntxin-4 have been shown to be important components in the exocytotic machinery in eosinophils (Hoffman et al., 2001; Logan et al., 2002), mast cells (Guo et al., 1998; Paumet et al., 2000), platelets (Chen et al., 2000), and neutrophils (Martin-Martin et al., 2000). Results from our lab have localized SNAP-23 and syntaxin-4 primarily to the plasma membrane, however, localization to the late endosomes and *trans*-Golgi have also been described (Logan et al., 2002). This suggests a role in vesicular trafficking. Further studies are needed to determine if this is the case in CTLs and NK cells. In this study, I have observed that SNAP-23, syntaxin-4 and syntaxin-6 were present in lysates of CTLs and NK cells. To determine the exclusivity of the localization further studies with membrane-enriched fractions is necessary. Based on previous findings (Logan et al., 2002), SNAP-23 and syntaxin-4 have been detected predominantly on plasma membrane fractions with minor immunoreactivity in the Golgi and ER. Based on Western blot data, it would appear that this could also be the localization of SNAP-23 and syntaxin-4 in CTLs and NK cells as lysates showed expression of both SNAP-23 and syntaxin-4 protein. This suggests localization to membrane fractions. More studies are needed to confirm this.

III. SNAREs and inflammatory cells

Inflammation is a response of the living tissue to injury and involves tissue infiltration of various inflammatory cells (Shukla et al., 2001). Recent studies and observations from our lab have shown SNAREs to be involved in exocytosis of other inflammatory cells. Various inflammatory cell types when stimulated in situ may influence and modify SL release in the pursuit of maintaining control of response to immunity and infection (Griffiths, 2002). The result of this may be harmful unless the mechanisms controlling the release of these mediators are tightly regulated. Recent studies have focused attention on members of the SNARE family of docking proteins in inflammatory and tissue-resident cells and a brief summary of the involvement of SNARE isoforms in granule secretion of other cells is discussed below and summarized in Table 5.

Neutrophils

Neutrophils have 4 distinct granule populations: secretory granules, primary, secondary and tertiary granules (Sengelov et al., 1993). SNARE complexes regulate neutrophil secretion in response to foreign invaders (Sjolin et al., 1994). SNAREs have been identified to secretory vesicles of neutrophils. The combination of RT-PCR and immunoblotting has shown the presence of syntaxin-4, VAMP-2, and SNAP-23 (Brumell et al., 1995; Nabokina et al., 1997). Ravichandran et al., 1996, have demonstrated in human neutrophils that SNAP-23 binds to multilple VAMPs and syntaxins *in vitro* (Ravichandran et al., 1996) and was important in docking and fusion. Human neutrophils were also found to express mRNA for multiple syntaxin isoforms including 1A, 3, 4, 5, 6, 7, 9, 11, and 16 (Martin-Martin et al., 1999).

VAMP-2 has been described (Brumell et al., 1995; Mollinedo et al., 2003) in neutrophil granule populations. This agrees with data generated from our lab by Logan et al., 2003, which localizedVAMP-2 to secondary and tertiary granules. Additionally, our lab has also found VAMP-7 to be involved in exocytosis of nuetrophil granules (secondary, tertiary and azurophilic) suggesting that VAMP-7 is critical for the trafficking of more than one granule compartment.

Mast cells

Mast cells are highly regulated and release granule contents upon stimulation. Thus far immunoblotting, cell fractionation and immunofluorescence has confirmed the presence of syntaxin-4 and SNAP-23, and VAMP-2 and syntaxin-3, in association with plasma membrane and granules, respectively (Guo et al., 1998). Additionally, VAMP-2, 3, 7, and 8 as well as syntaxin-2, 3, 4, and SNAP-23 have been reported in granules of a mast cell–like cell line, rat basophilic cell line, RBL-2H3 (Hibi et al., 2000; Paumet et al., 2000). The localization of SNAP-23 and syntaxin-4 has mainly been to the plasma membrane, while VAMP-2 has been identified as a granular protein (Guo et al., 1998). Studies from our lab have identified syntaxin-3, -4, -6, and SNAP-23 in the mast cell line LAD. Functional data is still not available using tissue mast cells. Regulation of the release of pro-inflammatory mediators from mast cells is important and therefore SNAREs may be important regulators of this process.

Macrophages

With a primary role of defence against pathogens, the identification of functional SNAREs in macrophages presents an important element of functional regulation. Syntaxin-2, 3 and 4 have been identified in plasma membranes by immunoblotting

(Hackam et al., 1996). VAMP was also detected in macrophages (Pitzurra et al., 1996), but VAMP-2 and 3 have been identified to be involved specifically in exocytosis of secretory vesicles (Bajno et al., 2000; Hackam et al., 1998). Further studies by Allen et. al., showed no marked impairment of maturation when VAMP-3 was deficient in mice (Allen et al., 2002), suggesting a redundancy in SNARE function.

Eosinophils and basophils

Host defense is an important role of eosinophils and basophil, thus the tight regulation of granule release is necessary. SNARE proteins play and important role in this regulation. The first indication came from studies involving guinea pig eosinophils, however it was found that VAMP-2 immunoreactivity was not detected and therefore may not play a role in membrane fusion of eosinophil crystalloid granules (Lacy et al., 2001). Results from our lab have shown by RT-PCR, immunoblotting and functional assays that SNAREs are indeed important in granule release from eosinophils Thus far VAMP-2, 7 and 8 as well as syntaxin-4 and SNAP-23 have been identified in eosinophils (Logan et al., 2002; Logan et al., 2003) by using similar techniques as this project. VAMP-7 was demonstrated to play a major role in exocytosis of large and small granules in human eosinophils as it was localized to $CD63^{+}$ compartments and to membrane-enriched fractions. t-Q-SNARE docking partners have been identified to be syntaxin-4 and SNAP-23. Preliminary data from the Moqbel lab on basophils have also suggested a similar profile of SNAREs. The studies on basophils continue to be developed.

Lymphocytes

Very little work has been done on lymphocytes with respect to SNARE proteins and their involvement in the regulation of the release of lymphocyte granules. My

project may be a stepping stone towards understanding lymphocyte exocytosis. Thus far, I have detected in CTL and NK cells VAMPs 1, 2, 3, 7, and 8 and syntaxins 3, 4, 6, and SNAP-23 by RT-PCR. Immunoblotting and immunofluorescence have confirmed protein expression for VAMP-7, 8 syntaxin 4 and 6 and SNAP-23 in CTLs and in NK cells, VAMP-7, syntaxins 4 and 6 and SNAP-23 have been confirmed. Interestingly, lysates of NK and CTLs did not show protein expression for VAMP-2 in either cell, nor VAMP-8 was expressed in cell lysates of NK cells. SNAREs seem only to partially colocalize with GrB, however GrB completely colocalizes with the SL marker CD63 in immunocytochemistry studies. While the presence of these proteins in the cells presupposes some function, we can not yet clearly state what that may be, nor can we speculate at this time which SNAREs are the most critical in SL secretion from CTLs and NK cells. In fact, it may be that exocytosis of CTL and NK granules may involve not only the SNARE-complex, but also other upstream regulatory mechanisms.

Table 5 - Presence of SNARE-Proteins in Inflammatory Cells

Cell Type	t-/Q-SNARE	v-/R-SNARE
Neutrophil	Syntaxin-3 Syntaxin-4 Syntaxin-6 SNAP-23	VAMP-1 VAMP-2 VAMP-7 VAMP-8
Mast Cell	Syntaxin-2 Syntaxin-3 Syntaxin-4 SNAP-23	VAMP-2 VAMP-3 VAMP-7 VAMP-8
Macrophage	Syntaxin-2 Syntaxin-3 Syntaxin-4 Syntaxin-6 SNAP-23	VAMP-2 VAMP-3 VAMP-7 VAMP-8
Eosinophil	Syntaxin-3 Syntaxin-4 SNAP-23	VAMP-2 VAMP-7 VAMP-8
Basophil	Syntaxin-4 Syntaxin-6 SNAP-23	VAMP-2 VAMP-3 VAMP-7 VAMP-8
CTL	Syntaxin-3 Syntaxin-4 Syntaxin-6 SNAP-23	VAMP-1* VAMP-2* VAMP-3* VAMP-7 VAMP-8
NK cell	Syntaxin-3* Syntaxin-4 Syntaxin-6 SNAP-23	VAMP-1* VAMP-2* VAMP-3* VAMP-7 VAMP-8

*-mRNA detected only. (Logan et al., 2003; Shukla et al., 2001)

IV. Rabs

The Rab proteins examined included Rab3d, Rab4a, Rab5a, and Rab27a. These Rabs were originally selected for examination because of their potential role in exocytosis. On further examination, Rab3d has been shown to be found predominantly in adipose cells (Fischer von Mollard et al., 1994). This suggested that the main function of Rab3d might be to interact with vesicle-membranes. Rab3d has also been shown to have no effect on mast cell degranulation, neither directly nor indirectly suggesting Rab3d may function as a down regulator of secretory granules before vacuole formation. The current view predicts that in non-neuronal cells, Rab3d exerts a stimulatory role in exocytosis rather than having direct involvement in the docking and fusion process, which are the steps we are primarily concerned with. A potential role for Rab3d in CTLs and NK cells may be granule maturation, which stems from results by Riedel *et al.*, 2002 who performed experiments with Rab3d knockout mice. They showed that mast cell degranulation was not reduced, although the volume and the size of the granules did show significant increases (Riedel et al., 2002).

Numerous studies, especially by Griffiths, have implicated Rab27a involvement in CTL granule secretion (Stinchcombe et al., 2001). Rab 27a deficient mice were found to be unable to secrete their granule contents such as hexosaminidase and granzymes. In these mice, while polarization does occur, the granules do not appear to dock. The role of Rab27a seems to be in the docking process and further examination is needed.

V. Summary and Implications of SNARE-Mediated Exocytosis in CTLs and NK Cells

Results stemming from this research project have suggested a potential role for SNARE proteins (and Rabs) in the exocytosis of cytotoxic granules from CTLs and NK cells. While expression and localization of key SNARE molecules was determined, the functional importance of these molecules in exocytosis remains unknown. Unfortunately, My attempts to induce artificial secretion of these cells by introducing Ca^{2+} and GTPγS to SLO-permeabilized cells was unsuccessful and required further optimization. This technique may not work in CTLs and NK cells because of rapid loss of cell responsiveness following permeabilization. The data presented have shown VAMP-7 (TI-VAMP) to be a promising SNARE involved in exocytosis of CTL and NK granules since it exhibited the highest detectable colocalization with GrB and CD63.

It is important to ensure that cytotoicity of CTLs and NK cells remain highly regulated. Target cell killing though GrB needs to be controlled with a high degree of specificity. Although much is known about the steps leading to exocytosis of cytotoxic granule contents, the exact mechanisms used at the critical docking and fusion step remain elusive. SNARE proteins may be involved in the regulation of docking and fusion of $CD8^+$ CTL granules as well as NK cell granules.

Beyond SNARES, exocytosis is further regulated by small Ras-like GTPases belonging to the Rab family (Fischer von Mollard et al., 1994; Zerial and McBride, 2001). Rabs provide a second level of specificity to the SNARE complex by matching the appropriate v-and t- SNAREs together (Zerial and McBride, 2001).

Thereby, understanding the basic mechanisms of exocytosis in $CD8^+$ CTLs and NK cells will aid in the development of novel therapeutic strategies that can be used to inhibit GrB release or similarly enhance its function. Thus, this study acts as a gateway For further research aiming at understanding and interfering with various conditions involving GrB including tumour rejection. Similarly, such studies may also be important in pathogenicities where GrB release is inimical and harmful including allograft rejection.

VI. Future Directions

While the observations made in this study so far are novel their implications remain uncertain until a functional role for SNAREs and Rabs has been determined in CTLs and NK cells. The primary future direction of this study is to determine the biological function of SNAREs. Below are proposals for the future studies.

1. Determine the functionality of SNAREs in the release of GrB from CTLs and NK cells using two techniques: SLO permeabilization and lenti-viral based transfection

SLO permeabilization

Permeabilization and stimulation of cells with GTPγS and Ca^{2+} has been suggested to be an effective method for inducing and measuring exocytosis of granules in various cell types since it permits the entry of exogenous proteins (including neutralizing antibodies) into the cell (Flaumenhaft et al., 1999; Lindau and Gomperts, 1991). Our lab, as well as others, have demonstrated this technique (Nusse and Lindau, 1990; Nusse and Lindau, 1993; Nusse et al., 1990) to be effective in both human neutrophils (Rosales and Ernst, 1997; Rosales and Ernst, 2000) and eosinophils. Although this technique has been successful in other cell types, this method of introduction of inhibitory antibodies into the CTLs and NK cells is limited, due to disruption of the cytoskeleton, loss of cell responsiveness, and the loss of cell integrity. Cell integrity is important to maintain, as CTLs and NK cells are triggered to secrete upon recognition of target cells at a cell-to-

cell level. To date, only few studies have explored at this technique in CTLs and NK cells mainly because it is complicated and difficult to applied to these two cell types. Although non-physiological stimulus may work, these do not represent the actual stimulation that occurs, *in vivo*. Therefore, identifying the tools to assess functionality of SNAREs and the role of candidate proteins involved in exocytosis is a difficult task Assessment of secretion would be through detection of β-hexosaminidase release (stored in SL of CTLs and NK cells) as applied in studied by Bonnema et al. 1994 as well as employing a GrB colorimetric assay to detect GrB secretion (Ewen et al., 2003).

Aizawa et al., 1992 have demonstrated that the secretory response of cells is dose-dependent on calcium (Aizawa et al., 1992). Therefore, numerous optimization studies will be needed in order to determine the dose of SLO and calcium, which induced granule secretion, but does not compromise cell integrity as triggering of maximal secretion is necessary in order to get inhibitory responses (Walev et al., 2001). FACS analysis of SLO-permeabilized cells will be used to determine the lowest concentration of toxin need to cause permeabilization of cells without compromising cell integrity.

I anticipate from observations in our lab that TI-VAMP- (VAMP-7) insertion into treated cells will cause a reduction in the release of β-hexosaminidase and GrB from CTLs and NK cells. From data I have already generated using RT-PCR, Western blot analysis and confocal microscopy, I would not expect significant amounts of reduction in release when VAMP-8 is inserted into cells as these v-/R SNAREs, which may suggest that this v-SNARE may not be associated with GrB-containing granules. It would be

interesting, however, to determine if VAMP-8 insertion affects the response in CTLs and YT-Indy.

Lenti-viral based transfection

This system will facilitate lentiviral-based regulated expression of my target genes (SNARE). The two main vectors are pLenti4/TO/V5-DEST, which is the destination vector to which the gene of interest is cloned, and pLenti6/TR vector, which contains *TetR* gene for constitutive expression of the Tet repressor.

Lentivirus works by entering the target cell, causing the viral RNA to be reverse transcribed. The RNA is then imported into the nucleus where it can be integrated into the host genome. Following tetracycline (Tet) addition, the target gene will be expressed. Tetracycline works to bind the Tet repressor and resulting in derepression of the promoter controlling the gene of interest. Selection of stable transfectants is through Zeocin, ampicillin and Blasicidin resistance genes in *E.coli* and mammalian cells.

Functional analysis and protein expression can be determined after DNA sequences have been entered into a vector system. This system is used because it is rapid, efficient, and relatively safe way to transfer DNA sequences into vectors. It is well known that lamba recombination occurs between special attachment sites (att) sites on the E.coli chromosome and on the lambda chromosome. The Gateway system we will be using incorporates many features to aid in all steps of the transfection procedure. Negative selection, following recombination and transformation, occurs through ccdB gene, allowing negative selection of donor and destination vectors in E.coli. presence of the ccdB gene does not allow growth of E.coli, however, once the gene of interest is inserted it will replace the ccdB gene and therefore E.coli will grow.

Overview

Our lab has designed a GFP (green-flourescnet protein)-syntaxin-4 construct with more still being developed. Our goal will be to over-express various SNARE proteins (VAMP-2, VAMP-7, VAMP-8, syntaxin-4, and SNAP-23) in order to inhibit GrB release from CTLs and NK cells. Overexpression of the protein will cause internal competition and therefore, itself will become non-functional. This GFP-construct will be overexpressed by insertion into a retroviral transduction system, pLenti6/V5 (Invitrogen). Viral superantent will be harvested from a 293FT producer cell line and selection of a ViraPower T-Rex cell line. A site-directed mutagenic strategy will be used to create dominant negative SNARE protein construct. The effects of these proteins on GrB release can be determined by analyzing target cell killing in a co-culture system using a chromium release assay and colorimetric assay for GrB.

2. Determine the presence and localization of VAMP-8 in inflammatory cells (neutrophils, eosinophils, mast cells, macrophages, basophils, CD4+ T-cells, B-cells).

It would be interesting to pursue such a study to determine if cells involved in inflammation have differential organelle-specific expression of VAMP-8. Differential expression and localization of the protein may suggest a cell specific role for VAMP-8 making VAMP-8 and attractive target for therapeutic manipulation. The techniques that should be implemented for this study are similar to the methods already employed in this thesis manuscript. RT-PCR and Western blot analysis would determine if different inflammatory cells do, in fact, express detectable levels of VAMP-8. Pending these results, localization of VAMP-8 can be determined using confocal microscopy and electron microscopy. Depending on localization, speculations can be made about the

function of VAMP-8 in each of these cell type. The finding of VAMP-8 in the nuclear area of YT-Indy already suggests a unique function for VAMP-8 in these cells. To compliment results, preparations of light membrane fractions as well as nuclear preparations should be examined by western blot analysis. These findings taken collectively can begin to piece together a model for VAMP-8 in exocytosis.

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Appendix A

PCR Reagents:

Superscript II RNase H Reverse Transcriptase kit includes: SuperScript II RT, 5X First strand buffer [250mM Tris-HCl, 375mMKCl, 15mM $MgCl_2$], 0.1 M DTT obtained from Invitrogen (Mississauga, ON). Taq DNA Polymerase, recombinant includes: Taq DNA polymerase, 10X PCR Buffer (200mM Tris-HCl, 500mM KCl), 50mM Magnesium Chloride obtained from Invitrogen (Mississauga, ON). dNTPs:100mM dATP, 100mM dCTP,100mM dTTP, 100mM dGTP were obtained from GibcoBRL and mixed in equal concentrations to make dNTPs.

PCR reaction temperatures:

VAMPs-1, 3, 7, 8: Step 1: 5 min at 94 ° C, Step 2: 45 sec at 94° C, Step 3: 45 sec at 60°C, Step 4: 2 min at 72°C, Step 5: repeat Steps 2-4 for 40 cycles, Step 6: 7 min at 72 °C, Step 7: 4 ° C for ∞.

VAMP-2: Step 1: 5 min at 94° C, Step 2: 45 sec at 94 °C, Step 3: 45 sec at 50 °C, Step 4: 2 min at 72 ° C, Step 5: repeat Steps 2-4 for 30-cycles, Step 6: 3 min at 72 °C, Step 7: 4 °C for ∞.

SNAP-23: Step 1: 5 min at 95° C, Step 2: 30 sec at 95° C, Step 3: 30 sec at 62°C, Step 4: 90 sec at 72°C, Step 5: repeat Steps 2-4 for 40-cycles, Step 6: 15 min at 72 °C, Step 7: 4 ° C for ∞.

Syntaxin-3, 4, 6: Step 1: 5 min at 94 ° C, Step 2: 1 min at 94 °C, Step 3: 1 min at 61 °C (57°C for Syntaxin-6), Step 4: 1 min at 72 ° C, Step 5: repeat Steps 2-4 for 40 cycles, Step 6: 3 min at 72 °C, Step 7: 4 °C for ∞.

Western blot Reagents:

RIPA: All cells were lysed in radioimmunoprecipitation (RIPA) buffer : 1 X PBS, 1% nonidet p-40 or Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS 10mg/mL PMSF in isopropanol (add 10ul/ml into RIPA). Running Gel Buffer: 1.5M Tris-HCL, pH 8.8. Stacking Gel Buffer: 0.5M Tris-HCl, pH 6.8. Running Buffer, 10X: 25mM Tris, 0.1% SDS (Sodium Dodecyl Sulphate), 192mM glycine, pH 9.3-8.9. Transfer buffer: 48mM Tris, 0.375% SDS, 39mM glycine, 20% methanol. SDS loading buffer: 187.5mM Tris-HCl, 6% SDS, 30 glycerol, 0.03% phenol red, 10% DTT (1.25). 10% acrylamide gel: 5mL 1.5 M Tris-HCl, 10.3 mL ddH2O, 5 mL 40% acrylamide (29:1), 200 μL 10% SDS, 18 μL tetramethylethylenediamine (TEMED). Transfer buffer: 50mM Tris, 40 mM glycine, 1.3 mM SDS, 20 % methanol.

SLO Reagents

0.25M $CaCl_2$: Dilute 1M of $CaCl_2$ (125ml 1M solution + 375ml dH_2O). 0.2M EGTA: Dissolve EGTA in 10M NaOH and 40mM PIPES. Adjust pH to 7. 1M Potassium Oxalate: 100mM ATP: in 0.2 M Tris. 10X Buffered Saline solution: 137mM NaCl, 27mM KCl, 2mM $MgCl_2$, 20mM PIPES in Baxter H_2O, pH = 6.8. Add 0.05g BSA to 1X BSS solution/50mL

SLO permeabilization of Cytotoxic T-cells and Natural killer cells

Ca^{2+}-EGTA buffer preparation

Materials

Potassium oxalate (1M 187.2g/L)

1 M $CaCl_2$ (73.5g in 500mL)

EGTA

PIPES (1M 302.37 mg/mL in 500mL)

50 mL burette

10mM ATP (0.55g in 10mL of 0.2M Tris base)

Method

- Prepare 500 ml 0.25 M $CaCl_2$ by diluting 1 M solution 1 : 4 (125 ml 1 M solution + 375 ml dH_2O).
- Prepare 500 ml of solution containing ~ 0.2 M EGTA + 40 mM PIPES. Dissolve EGTA by mixing acid form with 4x molar equivalent of NaOH (i.e. 0.4 moles or 20 ml 10 M NaOH in 500 ml). Adjust the pH to neutrality (pH 7).
- Clean all volumetric glassware (burette and pipettes) in detergent (Decon 90) or chromic acid. Rinse extensively in water. Before filling, rinse glassware in appropriate solution.
- Titrate 0.25 M $CaCl_2$ (in burette) with 50 ml buffered chelator after adding 5 ml of 10 M NaOH to keep pH above 10. Approximately 40 ml of $CaCl_2$ will have to be added.

Add indicator to the buffered chelator (2.5 ml 1 M potassium oxalate) and titrate until cloudiness persists for 1 min.

- Repeat this titration twice.
- Prepare the CaEGTA stock solution using same pipette and burette to mix 50 ml of buffered chelator (omit the alkali and indicator) with the exact same amount of $CaCl_2$ indicated by the titrations. The total volume should be approximately 90 ml.
- Prepare the EGTA stock solution by adding 40 ml water to 50 ml buffered EGTA.
- Adjust the pH of both solutions to 6.80 and bring their volumes up to exactly 100 ml in volumetric flasks.
- These two solutions may be stored in tightly capped Falcon tubes for many months at -20°C. Use the solutions in proportions shown in Table I to achieve the required level of free Ca^{2+}.

Table I
Recipe for Ca^{2+} buffer solutions

		Volume (ml)	
pCa **	[Ca]total [EGTA]total	CaEGTA	EGTA
8	0.014	0.112	7.888
7	0.124	0.996	7.004
6.5	0.310	2.481	5.519
6.0	0.587	4.698	3.302
5.5	0.819	6.552	1.448
5	0.938	7.501	0.499

Secretion assay for SLO permeabilized cytotoxic T-cells and Natural killer cells

Materials

Buffered salt solution (BSS), pH 6.8:

10X stock (500 ml):

137 mM NaCl	**40** g
2.7 mM KCl	**1** g
2 mM $MgCl_2$	**2** g
20 mM PIPES	**3** g or 10 mL 1 M PIPES

- **Adjust pH of stock to 6.8 with 10 M NaOH (do this slowly if using powdered PIPES, as PIPES takes time to dissolve). Check pH of diluted solution before use.**
- **Dilute solution 1:10 before use (usually make up 100 mL for each assay) and add 1 mg/ml BSA.**

EGTA solution (pH 6.8)

10 x 10^6 cells (10 mL) cell suspension/plate (density 1 x 10^6 cells/ml- 8 x10^6 cells/ml)

10% Triton-X-100 (in H_2O)

Streptolysin-O (100 μg/mL)

100 mM GTPγS (Boehringer Mannheim 95-97% pure solution)

Calcium buffers (*p*Ca 5 and 8) (made from protocol on previous page: see **)

Flat-well 96-well microplates (Falcon)

Remove from fridge:

10% TX-100 (in H2O) (for eosinophil lysates)

Remove from freezer:

100 mM ATP (may be frozen and thawed repeatedly)

Streptolysin-O (100 μg/mL) (**do not repeatedly freeze and thaw**)

Calcium buffers (pCa's can be frozen and thawed repeatedly)

100 mM GTPγS (may be frozen and thawed repeatedly)

Method

- Prepare 100 μM GTPγS by adding 1 μl frozen stock (100 mM) to 0.999 ml BSS buffer. Leave on ice.
- For neutrophil assays prepare 1% TX-100 solution in BSS (50 μl 1% TX-100 + 450 μl BSS). Leave on ice.
- Make up pCa 5 solutions (Table 2)

Table 2 - Tube Preparation for SLO permeabilization

Tube	GTPγS	BSS	pCa	100mM ATP
1	900ul	1.73mL	2.77ul (5)	90ul
2	900ul	1.73mL	2.77ul (5.5)	90ul
3	900ul	1.73mL	2.77ul (6)	90ul
4	900ul	1.73mL	2.77ul (6.5)	90ul
5	900ul	1.73mL	2.77ul (7)	90ul
6	0ul	2.63mL	2.77ul (8)	90ul

- Leave on ice.
- Prepare 2.0 μg/ml SLO solution. Leave on ice.

Microplate preparation

While spinning down 5 x 10^6 cells and resuspending these in 5 ml BSS, use a V-welled microtitre plate on ice to pipette following reagents.

- Resuspend cells in BSS at 1 x 10^6/ml. Do not leave cells in BSS any longer than 5-10 min as they gradually lose their secretory capacity.
- Remove pCa solutions from ice and allow these to warm at room temperature.
- Remove plate from ice and allow this to warm at room temperature.
- Get a reagent tray and adjust a multichannel pipette to 50 μl/well.
- Chill down some BSS to get it ready to stop the reaction.
- Pour cells into a reagent tray and quickly transfer 50 μl cells to all wells. Mix cells briefly to ensure mixing of SLO with cells.
- Place microplate into 37°C incubator for 2 min. The cells will be permeabilized in this step, so do not run over time!

- Remove microplate from incubator and transfer 50 µl of each pCa dilution into all wells (in replicate of 5).
- Incubate for 10 min at 37°C.
- Stop reaction by adding 100 µl ice-cold BSS to each well
- Centrifuge plate for 5 min at 1500 rpm (~350g) at 4°C
- Remove supernatants for analysis of secreted mediators (TX-100 lysed represent total granule-derived proteins)

β-Hexosaminidase assay (using fluorescent substrate)

Materials

Hex substrate : 4-Methylumbelliferyl N-acetyl-B-D-glucosaminide, Mw 379(Sigma M-2233)

Dissolve 1mM Hex substrate in 0.2M citrate buffer, pH 4.5 as follows:

37.94mg Hex substrate in 1.0mL DMSO (37.94mg/mL)

Add this to 99mL citrate buffer for a final dilution of 1mM Hex substrate

Filter before use (0.45μm)

Method

- Add 50μl sample (SLO permeabilized supernatant) to each well
- Add 50μl substrate solution to each well
- Incubate reaction for 2 hours at 37°C
- Stop reaction with 100μl 0.2 M Tris base
- Read plate in fluorescent plate reader at 450nm (excitation 356nm)

Colorimetric Assay for Granzyme B

Materials

2 X Reaction Buffer

100mM HEPES pH 7.5

20% sucrose

0.2%CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate)

Filter silution (0.22μm) and store up to 3 months at room temperature

Add 10mM DTT just before enzymatic assay.

Just before incubation add 1μl IEPD-pNA (200μl final concentration/50μl of 2X buffer)

Methods

- Add 51μl 2 X Reaction buffer
- Add 49μl of sample
- Total volume per well: 100μl
- Incubate reaction at 37°C for 1-3 hours
- Read at 405-410nm.