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Elucidation of the mode of action of the pore-forming toxin aerolysin on T lymphomas.

by

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B.Sc., Mount Saint Vincent University, 1995

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

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We accept this dissertation as conforming to the required standard

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ABSTRACT

Aerolysin is a channel-forming protein toxin secreted by virulent *Aeromonas* species. The toxin binds to receptors on cells, is proteolytically activated, and then assembles into a heptameric oligomer, which inserts into the plasma membrane forming a functional channel, resulting in cell death. To further characterize these steps receptor identification, the effect of membrane domains on channel formation and the mode of cell death were investigated on T lymphomas. Screening of cell lysates for proaerolysin-binding proteins N-glycosidase and phosphatidylinositol specific phospholipase C treatment and/or purification of these proteins resulted in the identification of a group of glycosylphosphatidylinositol (GPI)-anchored proteins, which included contactin, Thy-1, and placental alkaline phosphatase. Liposomes were used to show that these proteins were receptors for aerolysin as those containing Thy-1 or placental alkaline phosphatase in their membranes were at least 100-fold more sensitive to aerolysin than those without protein. Similarly, cells expressing GPI-anchored proteins were 10^4-fold more sensitive to aerolysin than cells lacking them. This is likely the result of these proteins concentrating aerolysin on the cell surface and thus promoting oligomerization. The fact that these proteins can be localized to membrane domains known as rafts, which are enriched in sphingomyelin and cholesterol has the potential to affect oligomerization. To investigate this possibility erythrocytes and T lymphomas were treated with methyl-β-

cyclodextrin, which destroys rafts by sequestering cholesterol. Raft disruption did not decrease the sensitivity of these cells to aerolysin. Similarly, aerolysin was no more active against liposomes containing placental alkaline phosphatase in raft domains than those in which the receptor was in non-raft domains. Thus raft domains do not promote channel formation by aerolysin. The mechanism of cell death was next investigated. At high toxin concentrations cell death was shown to proceed by necrosis, whereas at subnanomolar concentrations aerolysin triggers apoptosis. Using inactive aerolysin variants it was determined that apoptosis was not a result of binding to GPI-anchored proteins nor was it triggered by receptor clustering induced by oligomerization. Instead the formation of a small number of channels was shown to trigger apoptosis. Taken together these studies have helped to clarify the mode of action of aerolysin.

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TABLE

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LIST OF ABBREVIATIONS

Abbreviation	
AA	Aplastic anemia
ADP-RT	Adenosine diphosphate ribosyltransferase
APS	Ammonium persulfate
APT	Aerolysin pertussis toxin
BS3	bis (sulphosuccinimidyl)-suberate
BSA	Bovine serum albumin
cAMP	adenosine 3', 5' -cyclic monophosphate
CD	Circular dichroism
CDT	Cholesterol dependent toxin
CH	Cholesterol
CHO	Chinese hamster ovary
CF	Carboxyfluorescein
CRD	Cross reacting determinant
Cryl A	Insecticidal crystal protein toxin (*Bacillus thuringiensis*)
CT	Cholera toxin
D	Diffusion coefficient
DABCO	1,4-diazobicyclo [2.2.2] octane
DAF	Decay accelerating factor
DEAE	Diethylammonioethyl
dH_20	Deionised water
DMEM	Dulbecco's modified Eagle's medium

DMF	Dimethyl formamide
DTSSP	3,3'-dithiobis-(sulphosuccinimidylpropionate)
DT	Diphtheria toxin
EAR	Erythrocyte aerolysin receptor
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EF-2	Elongation factor-2
ELISA	Enzyme linked immunoabsorbant assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FLAER	Fluorescently-labeled aerolysin (ALEXA)
FRET	Fluorescence resonance energy transfer
Gal	Galactose
GalNAc	N-acetyl galactosamine
GlcN	N-glucosamine
GlcNAc	N-acetyl glucosamine
GPI	Glycosyl phosphatidylinositol
GPI-AP	Glycosyl phosphatidylinositol-anchored protein
HBS	HEPES buffered saline
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid])
HlyA	Hemolysin A
HlyE	Hemolysin E

HRP	Horse Radish peroxidase
kDa	KiloDalton
l_o	Liquid ordered
l_c	Liquid crystalline
MALDI TOF	Matrix-assisted laser desorption-ionization time of flight
Man	Mannose
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mV	Millivolt
NAD	Nicotinamide adenine dinucleotide
NANA	N-acetyl neuraminic acid
NCAM	Neural cell adhesion molecule
nm	Nanometer
OB	Oligosaccharide-binding
OD	Optical density
PC	Phosphatidylcholine
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PIG-A	Phosphatidylinositol glycan A
PI-PLC	Phoshpatidylinositol-specific phospholipase C
PLAP	Placental alkaline phosphatase
PMS	Phenazine methosulfate

PMSF	Phenylmethylsulfonyl fluoride
PNGase F	Peptide-N-glycosidase F
PNH	Paroxysmal nocturnal hemoglobinuria
proHB-EGF	Heparin-binding epidermal growth factor-like precursor
pS	PicoSimens
PT	Pertussis toxin
PVDF	Polyvinylidene fluoride
R domain	Receptor binding domain
rpm	Revolutions per minute
RTX	Repeats in toxin
SDS	Sodium dodecyl sulfate
SDS PAGE	SDS-polyacrylamide gel electorphoresis
SM	Sphingomyelin
SPR	Surface plasmon resonance
T domain	Translocation domain
TAE	Tris EDTA buffer
T_m	Transition temperature
Tris	Tris-(hydroxymethyl)aminomethane
Triton X-100	t-Octylphenoxypolyethoxyethanol
Triton X-114	Polyoxyethylene(8) isooctylphenyl ether
Tween 20	Polyoxyethylenesorbitan monolaurate
UV	Ultra violet
VSG	Variant surface glycoprotein

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DEDICATION

This thesis is dedicated to my mom and dad, Rose and Dave Nelson.

INTRODUCTION

More than 200 bacterial species are known to be pathogenic to humans or other animals. Inside mammals, pathogens find the ideal temperature, pH and nutrients for growth. However, to take advantage of these conditions, they must enter the body, lodge in a tissue, and multiply. To overcome host resistance pathogens use a variety of survival mechanisms; they escape from the immune system by invading and subsequently replicating inside host cells; they use superantigens to induce hypersensitivity reactions in the immune system; or they produce toxins that disrupt normal cellular functions (Finlay and Falkow, 1997; Proft *et al.*, 1998).

Defining the term toxin has proven to be difficult because of a lack of information on the modes of action of putative toxins and on their abilities to cause disease. In addition, compounds having toxic effects may be peptides, carbohydrates, lipids or proteins. The definition used here was proposed by Bonventre (1967), who suggested that the term toxin be restricted to disease-causing proteins of bacterial origin, which are characterized by high molecular mass, and antigenicity.

Protein toxins affecting mammalian cells can be divided into two major groups; those acting on intracellular targets and those acting at the cell membrane (Figure 1). Toxins interacting with intracellular targets have a diverse range of enzymatic activities and affect a variety of cellular functions. For example, adenosine diphosphate ribosylating (ADP-RT) toxins have been identified that

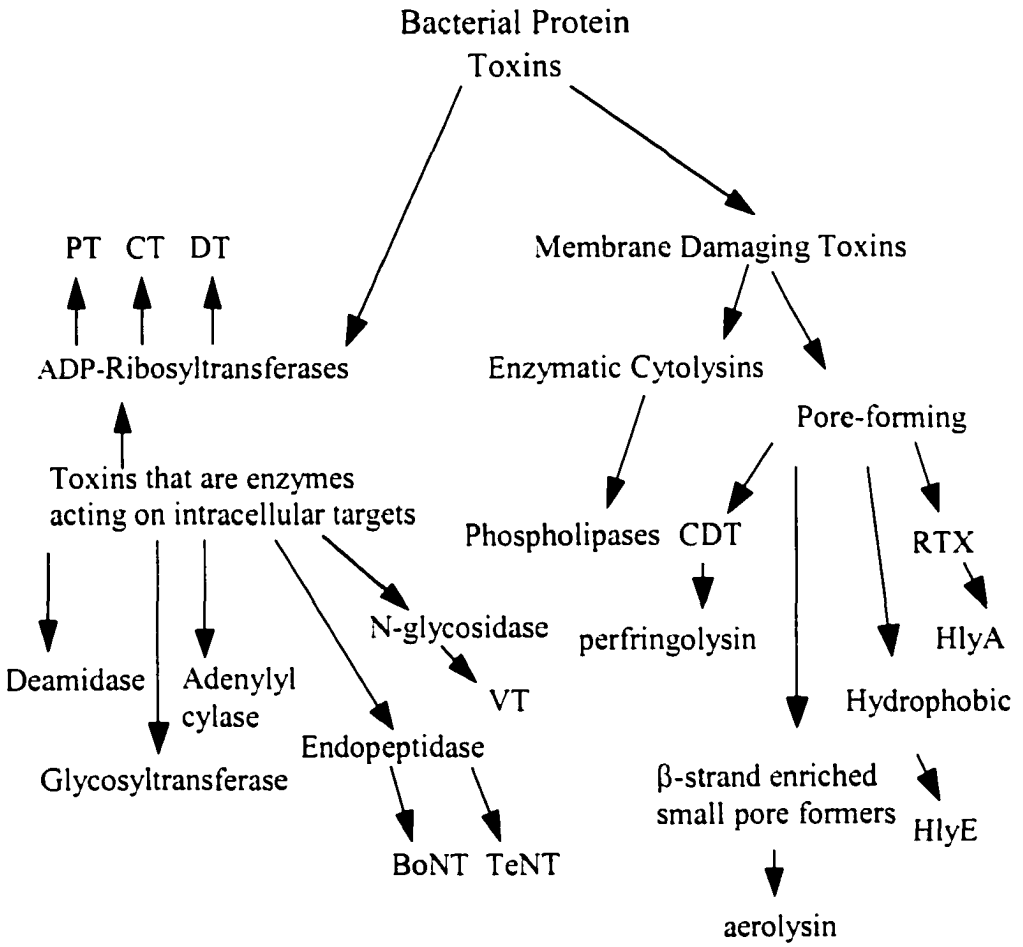


Figure 1: **Activities of bacterial protein toxins.**

affect proteins involved in the regulation of protein synthesis, actin polymerization, or adenylyl cyclase activity (Passador and Iglewski, 1994). Enzymatic toxins with glycosidase or glycosyltransferase activity modify GTP-binding regulatory proteins by the addition or removal of sugars (von Eichel-Streiber *et al.*, 1996). Other toxins disrupt neuroexocytosis by cleaving proteins involved in the vesicle fusion apparatus (Montecucco and Schiavo, 1993). Of the approximately 240 known bacterial toxins, approximately 40 % belong to the second group of toxins that act on the cell membrane (Braun *et al.*, 1991). This group includes lipolytic enzymes and pore-forming toxins. With the exception of a few interesting pore-forming toxins acting on insect or bacterial cells, the majority of the toxins discussed here will be those affecting mammalian cells.

The subject of this dissertation is the pore-forming toxin aerolysin, which is secreted by most virulent strains of *Aeromonas hydrophila* (Altwegg and Geiss, 1989; Buckley, 1999). Aerolysin forms channels in the membranes of a variety of mammalian cells including T lymphomas and erythrocytes (Nelson *et al.*, 1999; Howard and Buckley, 1982). This thesis deals with the interaction of aerolysin with T lymphomas. I will begin with a general introduction to bacterial toxins followed by an in depth look at aerolysin. The various cellular components and concepts involved in aerolysin mediated cytotoxicity will then be discussed.

Toxins that are known to be Enzymes with Intracellular Targets:

General Characteristics

Some toxins affect target cells by enzymatically modifying proteins involved in normal cellular function. The majority of these toxins are characterized by a common AB architecture, comprising an A domain that contains the enzymatic activity, and one or more B domains, involved in cell binding (Merritt and Hol, 1995). The A and B domains are typically either arranged with one catalytic A subunit and a pentameric receptor binding complex of five B subunits (hexameric AB_5 toxins), or in AB structure with a catalytic A domain, and a single B domain (AB toxins). The B domain of AB toxins is further subdivided into membrane translocation and receptor binding domains (Choe *et al.*, 1992). The catalytic A domain of AB toxins is directly disulfide linked to the B domain, whereas in hexameric AB_5 toxins it is disulfide linked to a linker region called A2, which associates with the B pentamer. Reduction of the disulfide bridge in both groups of toxins is required to release the enzymatically active A domain (Montecucco *et al.*, 1994).

In order to act intracellularly, toxins in this group must bind to the target cell, be internalized by vesicle-mediated endocytosis, insert into the vesicle membrane and fuse with the target membrane, or traverse the vesicle bilayer and be released into the cytoplasm. Only then can they modify their intracellular targets (Montecucco *et al.*, 1994). The AB and AB_5 toxins differ with respect to the mechanisms involved in these three steps. To illustrate these differences the mode of action of diphtheria toxin (DT), a well-studied representative member of

the AB group, and cholera toxin (CT), a representative of the AB_5 group, will be considered here.

Diphtheria toxin and Cholera Toxin

The ability of DT and CT to disrupt critical cellular functions makes them important virulence factors in the diseases diphtheria and cholera respectively. Diphtheria toxin is produced by *Corynebacterium diphtheriae* and is responsible for the hemmoraghic and necrotic lesions that occur throughout the body of an affected individual (Popovic *et al.*, 2000). Cholera toxin is produced by *Vibrio cholera*, and is responsible for the fluid loss and severe dehydration associated with cholera (Lai *et al.*, 1980, Richardson *et al.*, 1996). Both of these toxins possess ADP-RTs activity; however they have different structures, receptors, modes of translocation and intracellular targets. These differences will be used to illustrate the different modes of action of AB and AB_5 toxins.

Structure

The crystal structure of DT has been solved (Choe *et al.*, 1992). This toxin consists of a catalytic A domain that contains both α-helices and β-strands. These α- and β-structures come together to form a kidney shaped catalytic domain. The two lobes of this domain form a cleft, which contains the active site. The B domain of DT is further subdivided into a receptor binding domain (R), formed by a flattened β-barrel, and a membrane translocation domain (T). The T domain consists of a bundle of seven α-helices surrounding two more highly hydrophobic

α-helices (Choe *et al.*, 1992). This domain is disulfide linked to the A domain and is thought to facilitate its translocation into the cytosol (D'Silva and Lala, 2000).

The structure for CT is also known (Zhang *et al.*, 1995). In contrast to DT, it is a hexameric (AB_5) toxin divided into a catalytic A domain and a pentameric binding domain (B_5). Like DT the A domain of CT is in the shape of a kidney formed by α-helices and β-strands. The active site is located in a cleft between the two lobes of the domain (Zhang *et al.*, 1995). The catalytic A domain is attached to the binding domain via the linker A2, which is an extended α-helix. One end of the helix interacts with the A domain *via* a disulfide bridge and extensive hydrophobic interactions, while the other end inserts into the central channel formed by the β-pentamer (Zhang *et al.*, 1995). The β-pentamer consists of five identical binding subunits each containing an oligosaccharide-binding (OB) fold, which consists of two β-sheets that form a β-barrel capped by a long α-helix (Stein *et al.*, 1994).

Binding

Toxins are often produced in environments that are rapidly cleared, such as the gastrointestinal tract or bloodstream. In order to exert an effect before being flushed away, a toxin must bind to the cell surface. Diphtheria toxin uses a single domain to bind to a protein receptor (Choe *et al.*, 1992). This is the R domain of the B subunit, which uses its flattened β-barrel to bind to the protein receptor heparin-binding epidermal growth factor-like precursor (proHB-EGF). Since the binding of DT to proHB-EGF is a protein-protein interaction it has a high binding

affinity (K_D 2 x 10^{-8} M; Brooke *et al.*, 1998; Lanzrein *et al.*, 1996; Naglich *et al.*, 1992).

In contrast to DT, CT like most other AB_5 toxins, uses its pentameric binding domains to bind oligosaccharides on lipids positioning the catalytic domain close to the membrane (Minke *et al.*, 1999; Schon, and Freire, 1989). Although protein-carbohydrate interactions are usually of low affinity, since all five B subunits of CT contact a single molecule of ganglioside G_{M1}, a high binding affinity is generated (K_D 7 x 10^{-10} M; MacKenzie *et al.*, 1996).

Internalization

Following binding to receptors on the apical surface of epithelial cells, DT is internalized via clathrin-coated vesicles, whereas CT is internalized via uncoated vesicles (Falnes and Sandvig, 2000; Montecucco *et al.*, 1994). Diphtheria toxin crosses the membrane of late endosomes to exert its effect in the cytosol (Montecucco *et al.*, 1994), while CT is transported through the trans Golgi network and the ER on its way to its final destination, the basolateral membrane (Bastiaens *et al.*, 1996; Falnes and Sandvig, 2000; Lencer *et al.*, 1999).

Translocation

Following endocytosis, the catalytic A domain of DT crosses the endosome membrane, and is released into the cytoplasm where it comes into contact with its soluble target (Montecucco *et al.*, 1994). In contrast, the A domain of CT inserts into the endosome membrane and fuses with the basolateral plasma membrane to modify its membrane bound target (Falnes and Sandvig, 2000). Since these toxins are localized to different environments in the cell,

different translocation mechanisms are required to deliver them to their appropriate destinations.

The T domain of DT is critical to the translocation of the catalytic A domain into the cytosol. As mentioned above, the T domain consists of a bundle of seven α-helices surrounding two more α-helices that are highly hydrophobic (Choe *et al.*, 1992). The acidic environment found in late endosomes (pH 5.5) is thought to trigger changes in amino acid side chain protonation that catalyze unfolding of the bundle, exposing the two hydrophobic helices and allowing them to insert into the membrane (London, 1992). How insertion of the T domain into the membrane helps the A domain reach the cytosol is unknown; however, tunnel and cleft models have been proposed to explain A domain translocation (Montecucco *et al.*, 1994; Stenmark *et al.*, 1988). The controversial tunnel model proposes that the T domain creates a pore through which the A domain passes in an unfolded state (Boquet *et al.*, 1976). In the more widely supported cleft model, low pH causes a conformational change in the toxin that allows both the T and A domains to insert into the membrane. The T domain then generates a hydrophilic cleft that allows the A domain to cross the membrane with its hydrophobic segments exposed to the lipid bilayer and the hydrophilic segments contacting the cleft in the T domain (Montecucco *et al.*, 1992). Following translocation of the toxin into the cytosol, glutathione or another intracellular reducing agent reduces the disulfide bond between the A and T domains, releasing the A domain into the cytosol, where it exerts its enzymatic activity (Madshus *et al.*, 1994; Montecucco *et al.*, 1994).

Insertion of the A domain of CT into the membrane does not appear to involve a pH induced change in conformation as with DT (Menestrina *et al.*, 1994a). The pentameric binding subunits seem merely to position the catalytic A subunit close to the membrane, where it is then released from the binding subunit by disulfide bond cleavage (Olsnes and Sandvig, 1988). Following release, a hydrophobic portion of the A domain is exposed (Tomasi *et al.*, 1981). As a result, the free A domain is water insoluble, which favours its insertion into the vesicle membrane (Tomasi *et al.*, 1981). The A domain is inserted in such a way that upon fusion with the basolateral plasma membrane it is in a position to modify a trimeric G protein on the cytosolic leaflet of the membrane (Lencer *et al.*, 1999).

ADP-Ribosyltransferase (ADP-RT) Activity

Following translocation, DT and CT are finally ready to exert their enzymatic activities. As with several other enzymatic toxins, these two toxins catalyze ADP-ribosylation of target proteins essential for normal cellular function (Madshus *et al.*, 1994). ADP-ribosyltransferases transfer an ADP-ribose group from NAD^+ onto an acceptor protein, releasing nicotinamide (Madshus *et al.*, 1992). This is a classic covalent modification that alters protein function. Although DT and CT both possess ADP-RT activity, they have different intracellular targets and thus they have different effects on cells.

Diphtheria toxin exerts its activity in the cytosol where it targets elongation factor-2 (EF-2), a component of the protein synthesis machinery (Obrig *et al.*, 1994). Elongation factor-2 catalyzes the translocation of a tRNA

bound amino acid to the growing peptide chain on ribosomes. Diphtheria toxin ADP-ribosylates EF-2 at diphthamide, a postranslationally modified histidine residue (Wilson *et al.*, 1992). The addition of a bulky ADP-ribose group blocks EF-2 mediated translocation (Obrig *et al.*, 1994). Unable to synthesize new protein, the cell quickly dies. As a result, necrotic lesions form at affected sites in the body.

Unlike DT, which modifies a soluble target, CT modifies a membrane bound trimeric G protein (reviewed in Spangler, 1992). Trimeric G-proteins consist of G_α, G_β, and G_γ subunits associated with a transmembrane receptor (Sprang *et al.*, 1997). Following receptor activation by ligand binding, the G_α subunit binds GTP and dissociates from $G_{\beta\gamma}$ (Clapham *et al.*, 1996). Free G_α diffuses on the cytosolic face of the plasma membrane and binds to its effector molecule (Sprang *et al.*, 1997), in this case adenylyl cyclase (Spangler, 1992). The G_α subunit exerts its effect only temporarily, because its inherent GTPase activity hydrolyzes the bound GTP, causing inactivation of G_α, which dissociates from the adenylyl cyclase complex and binds $G_{\beta\gamma}$ again (Gierschik *et al.*, 1992, Wolff *et al.*, 1984). Stimulatory G-proteins contain a $G_{s\alpha}$ subunit that stimulates adenylyl cyclase, whereas inhibitory G-proteins contain a $G_{i\alpha}$ subunit that inhibits adenylyl cyclase.

ADP-ribosylation of the $G_{s\alpha}$ subunit by CT, blocks its inherent GTPase activity, thereby locking it in the active GTP bound state. Active $G_{s\alpha}$ continuously stimulates the adenylyl cyclase complex resulting in increased

cAMP levels (Spangler *et al.*, 1992). Elevated cAMP levels in enterocytes lead to inhibition of sodium channel function and activation of chloride channels. The resulting chloride loss and inability to reabsorb sodium cause diffusion-driven water loss from the cell (Spangler *et al.*, 1992). This results in diarrhea followed by the severe dehydration that is characteristic of cholera (Lai *et al.*, 1980).

Other AB toxins

In addition to the above mentioned enzymatic toxins, other well characterized and clinically relevant toxins deserve mention. Pertussis toxin (PT) is another well characterized AB_5 ADP-RT. Unlike other AB_5 toxins, which bind to gangliosides, PT appears to bind to a glycoprotein receptor (Stein *et al.*, 1996). Following internalization, PT targets trimeric G_i proteins, resulting in the disruption of cAMP levels (Stein *et al.*, 1996). Another enzymatic AB_5 toxin, whose devastating effects have recently been demonstrated in Walkerton, Ontario is verotoxin from pathogenic *E. coli*. This toxin binds to G_{b3} on intestinal epithelial cells and is internalized *via* clathrin coated pits (Cooling et al., 1998). The adenine N-glycosidase activity of verotoxin removes adenine 4324 from the 28S ribosome of the 60S rRNA subunit (O'Brien *et al.*, 1992). This modification interrupts protein synthesis resulting in the death of intestinal epithelial cells and diarrheal disease. The toxin also targets the kidneys resulting in haemolytic uraemic syndrome (Karmali, 1989; O'Brien *et al.*, 1992). Two other AB toxins that deserve mention are two of the most lethal substances known, tetanus and botulinum neurotoxin (Montecucco and Schiavo, 1993). These toxins bind to

neurons *via* a ganglioside receptor (Kozaki, *et al.*, 1998). Following internalization, they exert their endopeptidase activity on components of the neuroexocytosis apparatus involved in the release of neurotransmitters (Montecucco and Schiavo, 1994; Schiavo *et al.*, 1992). This results in paralysis, and death is often the result of suffocation due to the inability to control breathing (Ahnert-Hilger and Bigalke, 1995).

Cytolysins: bacterial proteins acting on cell membranes

The enzymatic toxins discussed above affect intracellular targets. An alternative target for other toxins is the plasma membrane of eukaryotic cells, which can be disrupted by lipolytic enzymes or spanned by a variety of pore-forming proteins. Lipolytic enzymes affect cells by degrading individual lipid molecules causing disruption of the plasma membrane, whereas the pore-forming toxins insert channels into the plasma membrane (Braun *et al.*, 1991; Titball, 1993). An overview of the activities of these two types of toxins will now be given followed by a detailed look at representative members. Special attention will be given to the pore-forming toxins as this is the group of toxins to which aerolysin belongs.

Enzymatic cytolysins

Many bacteria produce enzymes that hydrolyze lipids in the plasma membranes of target cells, often causing cell lysis. The majority of these bacterial enzymes hydrolyze phospholipids and thus are called phospholipases (Titball,

1993). Phopholipases are designated according to the site at which they cleave specific phospholipids (Nelson and Cox, 2000). The phospholipases A hydrolyze one of the two ester bonds attaching acyl chains to the glycerol backbone, resulting in the release of an acyl chain. Phospholipase A1 cleaves the 1-acyl ester while phospholipase A2 cleaves the 2-acyl ester. Individual phospholipases that cleave both of these ester bonds are called phospholipases B. Phospholipases C target the glycerophosphate bond resulting in release of the phosphorylated headgroup from the lipid, whereas phospholipase D cleaves the bond on the other side of the phosphate resulting in the release of just the headgroup. Like phospholipases C, sphingomyelinases catalyze the release of the phosphorylated headgroup from sphingomyelin (Titball, 1993).

A variety of phospholipases possessing the above mentioned enzymatic activities are released by a variety of Gram-negative and Gram-positive bacteria as single polypeptide chains ranging in molecular mass from 20-70-kDa (Titball, 1993). Two examples of these enzymes that will be considered here are phospholipase C from *Clostridium perfringens* and phospholipase A2 from *Vibrio parahaemolyticus* (Nagahama *et al.*, 1998; Shinoda *et al.*, 1991).

Structure

The crystal structure of phospholipase C from *C. perfringens* has recently been solved (Naylor *et al.*, 1999). This toxin can be divided into two domains; the C-terminal domain, which appears to be involved in binding and the enzymatic N-terminal domain. The C-terminal domain consists of two four-stranded β-sheets containing a calcium-binding domain. The structural similarity between this

domain and the eukaryotic calcium and phospholipid binding domain C2 found in vesicle fusion proteins among others, has lead to the suggestion that this domain is involved in binding (Naylor *et al.*, 1999). In contrast to the binding domain, the enzymatic domain consists entirely of α-helices. The active site of the enzymatic domain is located at the base of a channel that is large enough to accommodate a phospholipid molecule (Naylor *et al.*, 1999). The crystal structure of phospholipase A2 from *V. parahaemolyticus* on the other hand is not yet solved, however it may share structural similarities with other phospholipases A2. These enzymes also contain a C-terminal calcium-binding domain and an enzymatic N-terminal domain (Dessen *et al.*, 1999). Here again the N-terminal domain contains the active site located in a channel that accommodates a phospholipid molecule (Dessen *et al.*, 1999).

Enzymatic Activity

The lytic activity of phospholipases depends upon the class of lipid targeted, the bond hydrolyzed and the distribution of the lipid in the membrane (Titball, 1993). Phospholipases that are lytic permeabilize the membrane by degrading a class of lipid molecules that are abundant in the membrane; or by generating products that solubilize the membrane. For example, phospholipase C from *C. perfringens* is lytic as it cleaves phosphatidylcholine (or sphingomyelin), which together account for 50% of the total membrane lipid (Alberts *et al.*, 1994). Hydrolysis of these abundant lipids generates large amounts of phosphocholine and diacylglycerol (or ceramide) resulting in a destabilized membrane and subsequent membrane rupture (Naylor *et al.*, 1999). Alternatively, phospholipase

A2 from *V. parahaemolyticus* exerts its lytic activity by the production of fatty acids and lysophospholipids that act as detergents, which solubilize the lipid bilayer (Menestrina *et al.*, 1994a).

Pore-forming Toxins

In contrast to the enzymatic toxins that disrupt the lipid bilayer by degrading it, pore-forming toxins disrupt membrane permeability by inserting a protein channel into the membrane. Pore-forming toxins may or may not require activation prior to binding to the cell surface. Once bound, these toxins undergo a transformation to generate a channel, which disrupts the cell membrane ultimately resulting in cell death (Braun *et al.*, 1991).

Activation

Pore-forming toxins are secreted as water-soluble proteins that may or may not require activation. Bacteria that release toxins in an inactive form likely do so to protect themselves from lysis. These proteins may be subsequently activated by proteolytic cleavage of an activation peptide. For example, aerolysin and *Clostridium septicum* α-toxin are activated by cleavage of a C-terminal peptide that is carried out by soluble or membrane bound proteases (Ballard *et al.*, 1993; Howard and Buckley, 1985). An alternative activation mechanism involves acylation of internal lysine residues (Stanley *et al.*, 1994). This mechanism is employed by *E. coli* HylA (Stanley *et al.*, 1998).

Binding

As with enzyme toxins, in order to exert an effect on a cell before being flushed away in the gastrointestinal tract or the bloodstream, pore-forming toxins must bind to the surface of target cells. This is accomplished by the use of a protein, or lipid receptor. For example, aerolysin and *C. septicum* α-toxin bind to GPI-APs, whereas *S. aureus* α-hemolysin binds directly to lipid head groups (Diep *et al.*, 1998a; Cowell *et al.*, 1997; Ellis *et al.*, 1997; Gordon *et al.*, 1999; Nelson *et al.*, 1997). For some toxins, including aerolysin, both inactive and active forms are able to bind to receptors on target cells (MacKenzie *et al.*, 1999). Toxins that bind in an inactive form are subsequently activated by cell associated proteases. Binding of active toxin molecules to cell surfaces concentrates them close to the plasma membrane for the next step, which is channel formation.

Channel formation

Following binding, active toxin molecules on the cell surface undergo a transformation from a water-soluble form to an amphiphilic form that is able to span the membrane. Pore-forming toxins employ several mechanisms to form membrane spanning channels from soluble proteins. One mechanism, employed by HlyA, involves the use of hydrophobic or amphipathic α-helices that are sequestered in the core of the protein, which become exposed to span the membrane generating a channel (Menestrina *et al.*, 1994a). Another more widely used mechanism of transformation proceeds by the oligomerization of toxin monomers to create a polymeric pore (Parker *et al.*, 1994). For example, HlyE from *E. coli* contains hydrophobic patches that come together in such a way that

the hydrophobic domain of each monomer interacts with the bilayer (Wallace *et al.*, 2000). Other toxins do not contain hydrophobic domains capable of spanning the bilayer. Instead they undergo differing degrees of structural rearrangement to form oligomeric β-barrels capable of spanning the membrane. For example, seven *S. aureus* α-hemolysin monomers expose an amphipathic β-hairpin to form a 14 stranded β-barrel capable of spanning the membrane (Song *et al.*, 1996).

The channels generated by the different mechanisms vary in composition from possibly monomeric HlyA, heptameric aerolysin and α-hemolysin oligomers and octameric HlyE, to 35-50-member oligomers for perfringolysin (Menestrina *et al.*, 1994a). Correspondingly, the diameters of the channels generated vary in size from 1 nm for HlyA, 1-3 nm for heptameric oligomers, 2.5-3 nm for octameric HlyE, to 35-50 nm for perfringolysin channels (Menestrina *et al.*, 1994a; Morgan *et al.*, 1994; Palmer *et al.*, 1998; Parker *et al.*, 1996; Wallace *et al.*, 2000). Small channels allow for the passage of ions, water and other small molecules across the membrane, whereas large 35-50 nm pores allow proteins and other large molecules to leak out of the cell (Braun *et al.*, 1991; Bhakdi *et al.*, 1996).

Cell death: necrosis *vs.* apoptosis

The movement of small molecules and ions across the membrane through small diameter channels creates an osmotic imbalance that results in the uninhibited flow of water into the cell, causing swelling and eventual cell lysis (Braun *et al.*, 1991). Cell death resulting from the formation of large diameter channels is due to the massive loss of intracellular contents (Bhakdi *et al.*, 1996).

In both cases cell death has generally been thought to proceed by necrosis. However, at low concentrations of HlyA and α-hemolysin (two toxins forming small diameter channels), cell death has been shown to proceed by apoptosis (Chen and Zychlinsky, 1994; Martin *et al.*, 1990; Muller *et al.*, 1999). The induction of cell death by either necrosis or apoptosis will now be discussed.

Necrosis is caused by irreversible damage to plasma or organelle membranes, overwhelming the cell, which then disintegrates (Thompson, 1998). Since necrosis is a passive process in which the cell does not participate in its own death, the intracellular contents spill out inducing a localized inflammatory response. In contrast to necrosis, apoptosis is an active process in which the cell participates in its own death. Apoptosis, or programmed cell death is a carefully orchestrated suicide mechanism in which the cell activates an intracellular cascade resulting in the systematic dismantling of cellular components (Arch and Thompson, 1999). The degraded cellular components are tidily packaged into apoptotic bodies that in turn are engulfed by other cells, thus avoiding an inflammatory response (Golstein, 1998).

Apoptosis can be induced by a variety of mechanisms; one of the best characterized is binding to specific receptor molecules on the cell surface. Two well characterized transmembrane receptors involved in the induction of apoptosis are the tumor necrosis factor receptor and CD95 (Ashkenazi and Dixit, 1998). These transmembrane receptors each contain an extracellular ligand-binding domain and an intracellular death domain that associates with components of the apoptosis machinery. Upon extracellular ligand binding, the

intracellular death domains of these receptors are activated, enabling them to interact with the apoptotic machinery. These interactions result in the activation of caspases, a key set of proteases involved in the subsequent induction of the apoptotic signaling cascade (Ashkenazi and Dixit, 1998). Binding and crosslinking of other surface receptors such as Thy-1 on T lymphomas has also been shown to trigger apoptosis, although the mechanism of induction is yet to be elucidated (Hueber *et al.*, 1994). Another mechanism for the induction of apoptosis alluded to above is the formation of a small number of channels by bacterial toxins. In this case, apoptosis may be due to an influx of calcium from the extracellular fluid, which may activate the calcium dependent protease calpain, which has been implicated in apoptosis (Duke *et al.*, 1994; Jones *et al.*, 1989; Squier *et al.*, 1994).

The study of the induction of apoptosis or necrosis by bacterial toxins involves looking for characteristic biochemical and morphological changes. These include caspase and calpain activation (Casciola-Rosen *et al.*, 1994; Kothakota *et al.*, 1997; Squier *et al.*, 1994; Thornberry and Lazebnik, 1998). In addition, morphological changes including cell shrinkage, membrane blebbing, phosphatidylserine exposure on the outer leaflet of the plasma membrane, condensation of nuclear material and the cleavage of DNA into internucleosomal subunits of 180-200 base pairs can be monitored (Ellis, 1991; Thompson, 1998; Wylie *et al.*, 1984).

Groups of pore-forming toxins

Pore-forming toxins can be divided into four main groups. These include the RTX toxins from Gram-negative organisms, of which *E. coli* HlyA is a representative member, a novel group of hemolysins from Gram-negative organisms that appear to use a hydrophobic patch to insert into the membrane including *E. coli* HlyE, cholesterol-dependent toxins (CDTs) from Gram-positive organisms including perfringolysin from *C. perfringens*, and a disparate group of toxins produced by both Gram-positive and Gram-negative bacteria that contain extensive beta structure and form small pores (Parker *et al.*, 1996; Rossjohn *et al.*, 1997b; Song *et al.*, 1996; Stanley *et al.*, 1998). This last group includes the well characterized aerolysin from *A. hydrophila*, α-toxin from *C. septicum*, and α-hemolysin from *S. aureus* (Coote *et al.*, 1992; Parker *et al.*, 1996; Welch, 1991; Welch *et al.*, 1992).

RTX toxins

The RTX toxins produced by *E. coli* and a variety of other Gram-negative bacteria including *Proteus vulgaris* and *Pasteurella haemolytica*, form a unique group of 102-177-kDa proteins that must be acylated to become active (Lally *et al.*, 1999). How acylation activates these toxins is not known for certain, however the acyl chains may be involved in anchoring the toxin to target cell membranes prior to channel formation (Stanley *et al.*, 1998). This family was so named because of the presence of a nine amino acid consensus sequence that is repeated between 6 and 40 times within each toxin (Repeats in ToXin; Strathdee *et al.*,

1989). A representative member of this family is HlyA from *E. coli*, which will be considered in detail below (Menestrina *et al.*, 1994b).

Structure

HlyA is a 110-kDa protein that contains two internal acylated lysine residues separated by approximately 100 residues (Lally *et al.*, 1999). Sequence analysis of HlyA reveals that it contains an internal calcium-binding domain and an N-terminal domain containing at least 10 hydrophobic or amphiphilic α-helices approximately 20 residues long (Coote, 1992; Lally *et al.*, 1999). This N-terminal domain appears to share structural homology with the pore-forming domain of colicin A, a channel forming toxin targeting bacterial cells. For this reason this domain of HlyA is suggested to be involved in channel formation (Menestrina *et al.*, 1994a).

Binding

Both the calcium-binding domain and the acyl chains of HlyA have been proposed to be involved in binding to cell surfaces (Coote, 1992; Stanley *et al.*, 1998). In fact it has been suggested that calcium binding is the trigger for the exposure of the acyl chains, making them available for membrane binding and insertion as is seen for the calcium-dependent exposure of a myristoyl chain on recoverin (Ames *et al.*, 1997).

Another recently identified component involved in the binding of HlyA to the cell surface is the β-2 integrin LFA-1 found on most leukocytes. This integrin

functions as a receptor for HlyA; however the regions of the toxin involved in interacting with this receptor have not yet been identified (Lally *et al.*, 1997).

Channel formation

Although a mode of channel formation is yet to be fully elucidated for HlyA, the use of α-helices to generate membrane spanning domains has been well characterized for a variety of other proteins. For example, the translocation domain of DT uses hydrophobic α-helices to span the membrane. Colicin A from *E. coli* and the Cry 1a delta endotoxin are two *bona fide* pore-forming toxins that also use this mechanism (Parker *et al.*, 1990; Smedley and Ellar, 1996). It has been suggested that the transformation of HlyA from a water soluble to an insertion competent form involves the exposure of hydrophobic helices from the core of the toxin as is the case for the other toxins (Lally *et al.*, 1999). Binding to a surface receptor or the plasma membrane may trigger a structural change in the protein allowing for the exposure of these helices, although this has yet to be demonstrated (Lally *et al.*, 1999).

One molecule of HlyA appears to be sufficient for channel formation although the possibility that oligomerization can generate larger pores has not been completely ruled out (Crammer *et al.*, 1995). Planar lipid bilayer studies reveal that HlyA generates a 1 nm diameter cation selective channel, which disrupts the osmotic balance in the cell resulting in cell death by osmotic lysis (Menestrina *et al.*, 1996). It has also been demonstrated that at low concentrations HlyA is also able to induce cell death by apoptosis (Jonas *et al.*, 1993).

Hydrophobic patch toxins

The hydrophobic patch toxins are another family of closely related cytolysins released by Gram-negative bacteria including *E. coli*, *Salmonella typhi*, and *Shigella flexneri*. HlyE from *E. coli* is a representative member of this family (Ludwig *et al.*, 1999).

Structure

The crystal structure of HlyE reveals that each monomer contains four long α-helices, which come together to form a rod-shaped structure (Wallace *et al.*, 2000). At the end of this rod there is an additional structural element referred to as a β-tongue. This tongue acquired its name because of its β-strand composition and tongue-like shape and projection from the monomer. The tongue is composed of a hydrophobic β-turn consisting of 27 residues capable of interacting with the hydrophobic core of a lipid bilayer (Wallace *et al.*, 2000).

Binding

Although detailed structural information is available on HlyE. It is yet to be determined whether this toxin uses a receptor and/or which part of the toxin is involved in binding to the cell surface.

Channel formation

A model for how HlyE oligomerizes has been proposed based on information obtained from the crystal structure of the monomer and image analysis of 2-D crystals of the oligomer by electron microscopy (Wallace *et al.*, 2000; Oscarsson *et al.*, 1999). In this model, tongues of up to eight HlyE monomers come together to form a functional pore while the remainder of the rod

shaped α-helical bundle extends up away from the membrane (Wallace *et al.*, 2000). The hydrophobic tongue of each monomer is proposed to make extensive contacts with the hydrophobic core of the lipid bilayer following oligomerization and insertion, while the α-helices on the other side of each monomer contain hydrophilic residues that line the water filled pore (Wallace *et al.*, 2000).

As mentioned above the channel consists of eight HlyE monomers. This channel has an internal diameter of 2.5 to 3 nm (Ludwig *et al.*, 1999), which causes cell lysis. It is yet to be determined whether this toxin is also able to induce apoptosis.

Cholesterol-dependent toxins

Unlike the above two families, the cholesterol-dependent toxins (CDTs) are produced by Gram-positive bacteria, including species from *Streptococcus, Clostridium, Listeria* and *Bacillus* genera (Tweten *et al.*, 1995). The members of this family are homologous proteins secreted as single polypeptide chains ranging in size from 50 to 80-kDa. Perfringolysin is a representative member of this family for which the crystal structure has been solved (Rossjohn *et al.*, 1997b). It will be used to illustrate the properties of CDT's.

Structure

The structure of the perfringolysin monomer can be divided into four domains, reminiscent of the structural organization of the aerolysin monomer (Figure 3; Rossjohn *et al.*, 1997b; Sowdhamini *et al.*, 1997). Domain 3 is situated away from the rest of the molecule in a manner similar to domain one of

aerolysin, while domains 1, 2 and 4 form a structure corresponding to the large lobe of aerolysin (Sowdhamini *et al.*, 1997). Domain 3 contains both α-helices and β-strands, whereas domains 1, 2, and 4 contain almost entirely β-strands.

Binding

A tryptophan rich pocket at the base of domain 4 seems to be involved in binding to cholesterol in the target cell membrane (Nakamura *et al.*, 1995). Prior to binding domain 3 contains four β-strands and six α-helices (Rossjohn *et al.*, 1997b). Binding results in a rearrangement of domain 3, which appears to be the trigger for oligomerization. This rearrangement converts the six α-helices of domain 3 into β-strands. The conversion into a completely β-strand structure transforms domain 3 into two amphipathic β-hairpins (Shatursky *et al.*, 1999).

Channel formation

The two amphipathic β-hairpins generated upon binding insert into and span the membrane. The use of two amphipathic β-hairpins per monomer to form a membrane spanning domain is novel in that *S. aureus* α-hemolysin uses only one hairpin per monomer to form a β-barrel that spans the bilayer (Song *et al.*, 1996). It is not known how the two amphipathic β-hairpins from each monomer are used to form a functional channel, or whether additional structural elements are involved in channel formation. It is possible that the β-hairpins may come together in some kind of β-barrel conformation as seen for other pore-forming toxins (Shatursky *et al.*, 1999).

Between 30 and 50 perfringolysin monomers oligomerize into ring-shaped structures (Bhakdi *et al.*, 1985). These oligomers insert into membranes forming

channels with a large central pore whose internal diameter (depending on the number of subunits in the oligomer) ranges from 35 to 50 nm (Olofsson *et al.*, 1993). Their formation leads to massive solute loss causing cell death by necrosis (Bhakdi *et al.*, 1985).

Toxins using β-structure to form pores

Toxins enriched in β-structure which form small pores include a variety of cytolysins from both Gram-negative and Gram-positive bacteria. This group includes the well studied aerolysin from *A. hydrophila*, α-toxin from *C. septicum* and α-hemolysin from *S. aureus* (Coote *et al.*, 1992; Welch *et al.*, 1991; Welch *et al.*, 1992; Parker *et al.*, 1996). Although these toxins are enriched in β-strands, they do not contain hydrophobic or amphipathic domains long enough to span the bilayer. To overcome this, these toxins each oligomerize to generate a β-barrel, which is used to span the membrane. The crystal structure for the β-barrel containing oligomer of *S. aureus* α-hemolysin has been solved and so it will be considered as a representative member of this group of toxins (Song *et al.*, 1996).

Structure

Alpha-hemolysin exists in solution as a 33-kDa water-soluble monomer. Although only the crystal structure of the oligomer has been solved, far UV CD spectra indicate there are no major changes in secondary structure upon oligomerization. As a result the structure of the oligomer can be used as a model for the monomer (Song *et al.*, 1996). The structure reveals that each of the seven monomers making up the oligomer are composed almost entirely of β-structure.

The monomer is thought to have a compact domain. Only during channel formation is a glycine rich loop exposed, which will eventually form the membrane spanning channel (Song *et al.*, 1996).

Binding

Staphylococcus aureus alpha-hemolysin does not appear to use a protein receptor on the surface of the target cell, but rather interacts directly with the plasma membrane (Bhakdi *et al.*, 1991). Basic and aromatic residues are believed to interact with the negatively charged phospholipid headgroups facilitating binding to the cell membrane (Song *et al.*, 1996).

Channel formation

To transform itself from a soluble form to a membrane spanning form, the toxin must undergo a conformational change. Binding of monomers to the cell membrane may trigger strand movements, which facilitate the subsequent assembly of a heptameric prepore structure (Song *et al.*, 1996). A glycine rich loop exposed from each of seven protomers in the heptameric prepore, folds into an anti-parallel β-hairpin. These seven β-hairpins then come together to generate a 14-stranded amphiphilic β-barrel. This β-barrel penetrates the lipid bilayer forming a functional channel in the cell membrane (Song *et al.*, 1996).

The crystal structure of the heptameric channel reveals that it has a mushroom shape. The stem region of the channel contains the β-barrel that spans the bilayer while the cap region sits flush against the membrane (Song *et al.*, 1996). The interface between the stem and cap domain contains a crevice rich in basic and aromatic amino acids, which are believed to bind the negatively charged

phospholipid head groups (Song *et al.*, 1996). Extensive hydrophobic and hydrophilic interactions between regions of the cap domain and adjacent strands in the stem domain create a very stable complex (Song *et al.*, 1996). The hydrophobic exterior of the stem domain interacts with the hydrophobic acyl chains of surrounding lipid molecules, while hydrophilic residues line the 1 to 3 nm diameter fluid filled channel that runs down the center of the oligomer (Song *et al.*, 1996). The generation of these small diameter channels results in osmotic lysis at high concentrations and apoptosis at low concentrations (Bhakdi and Tranum-Jensen, 1991; Jonas *et al.*, 1994).

Aerolysin

Overview of mode of action of aerolysin

Aerolysin is secreted by virulent *Aeromonas* strains as an inactive dimer precursor called proaerolysin (Buckley, 1999). The toxin binds to receptors on the surface of target cells and is activated by proteolytic nicking near the C-terminus (Abrami *et al.*, 1998; Howard and Buckley, 1982). Once activated, aerolysin oligomerizes to form a seven-member oligomer that inserts into the cell membrane forming a functional channel (Figure 2; van der Goot *et al.*, 1993b). Evidence presented here will show that cell death as a result of channel formation can occur by either necrosis or apoptosis.

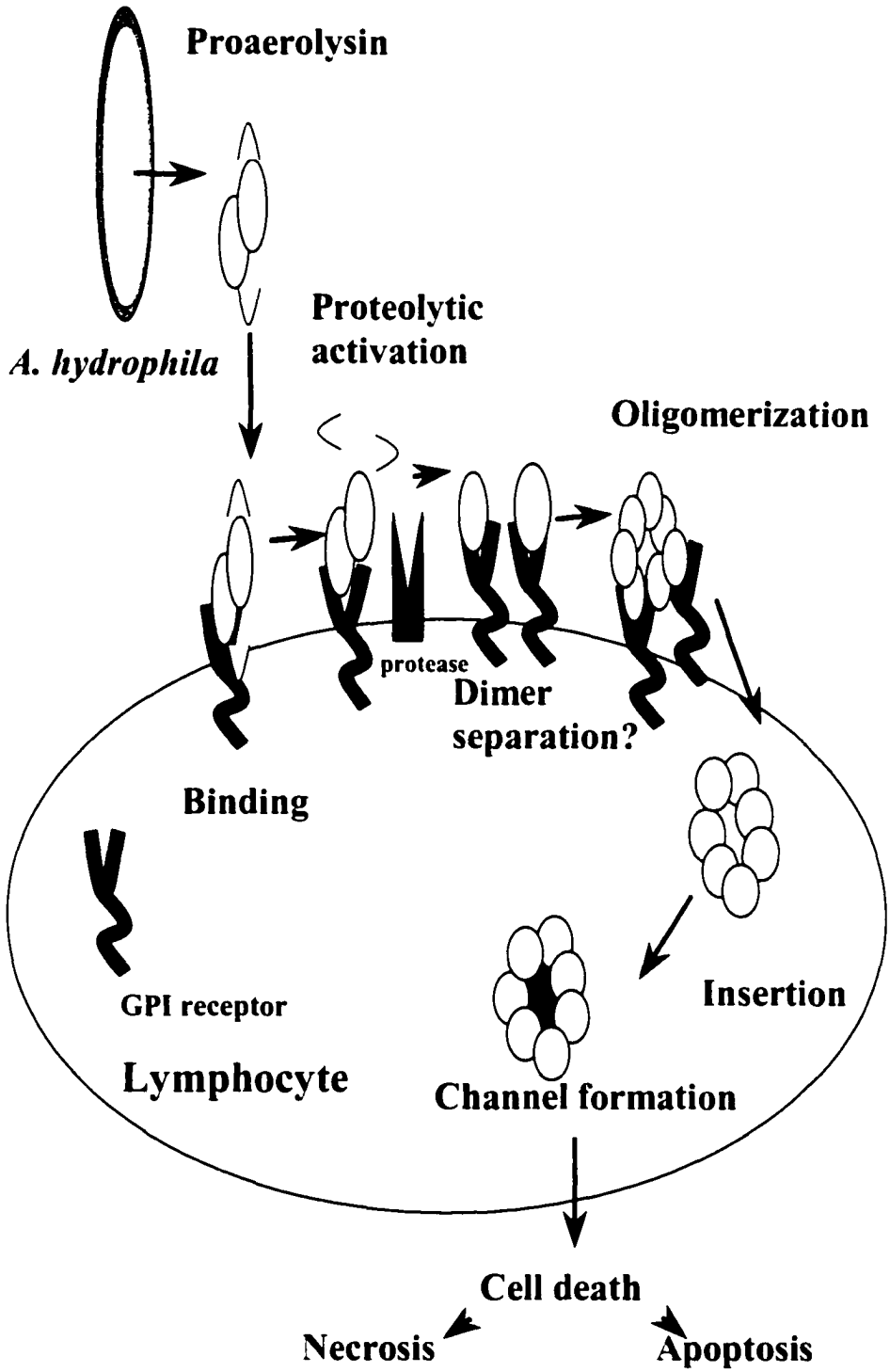


Figure 2: **Schematic of the mode of action of aerolysin**.

Toxin structure

Proaerolysin is one of the best characterized pore-forming toxins. This may be because it was the first for which the crystal structure was solved (Parker *et al.*, 1994). Aerolysin is a noncovalent dimer in the crystal. Each monomer is divided into two lobes; a small lobe also referred to as domain 1 and a large lobe divided into domains 2, 3 and 4 (Figure 3). The small lobe has a globular structure containing two 3-stranded anti-parallel β-sheets that form a pocket enclosing two α-helices (Rossjohn *et al.*, 1997a). The large lobe on the other hand is an elongated domain containing an extensive amount of β-structure, including some strands that run its entire length (Buckley, 1999). Regions of this lobe are involved in binding and channel formation (Diep *et al.*, 1998b; MacKenzie *et al.*, 1999).

Analysis of the crystal structure, chemical modifications, and site-directed mutagenesis of aerolysin have led to the identification of functional residues in the toxin. Each monomer contains four cysteine residues. Two of these cysteine residues are found in the small lobe, where they form a disulfide bridge, while the other two form a disulfide bridge at the top of the large lobe. A critical histidine residue (His 132) located in domain 2 is involved in oligomerization as its mutation to asparagine completely inhibits oligomerization (Wilmsen *et al.*, 1991). The mutation of two tryptophan residues (Trp371 or Trp373) to leucine on the other hand enhances the rate of oligomerization 10 to 20 fold (van der Goot et al., 1993b). Site directed mutagenesis has also confirmed that aromatic residues

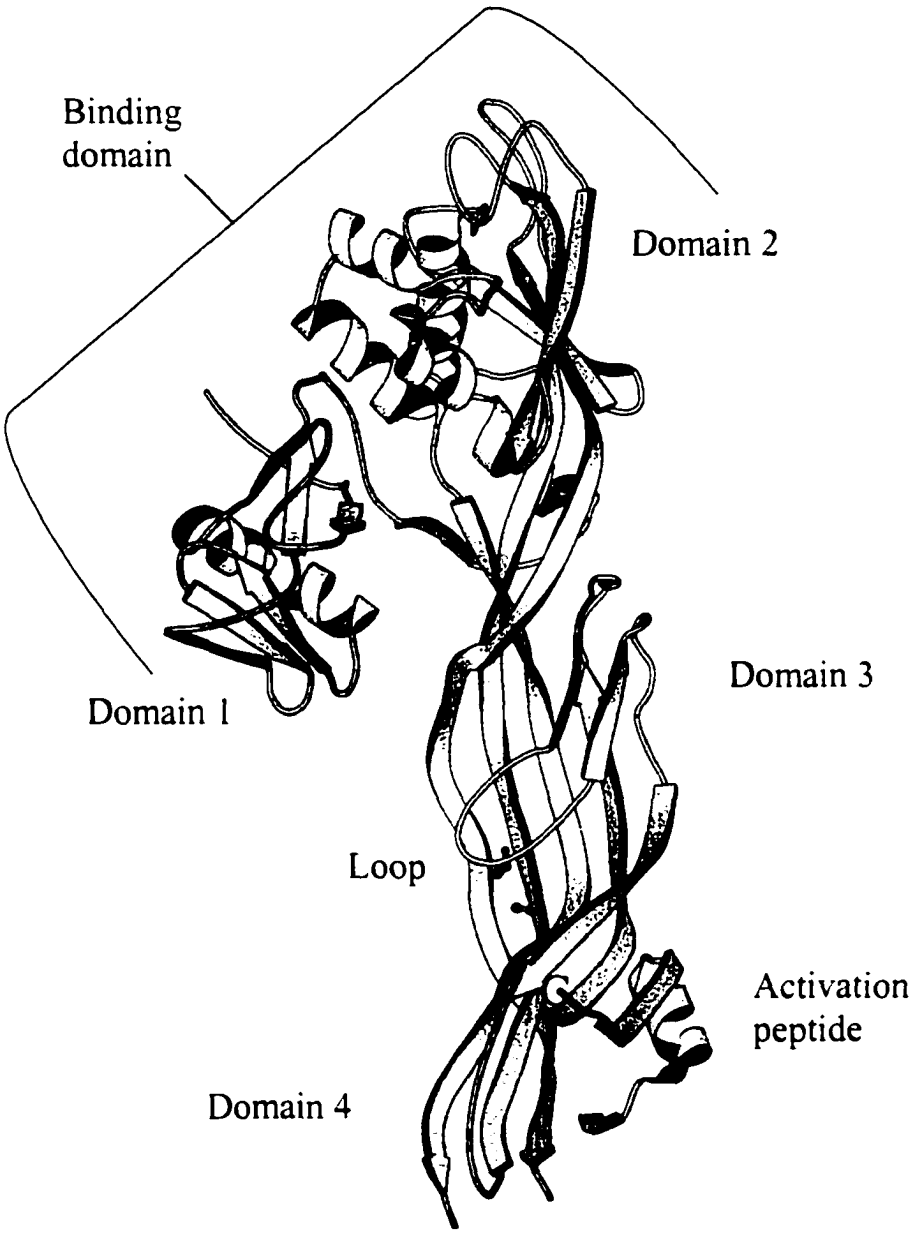


Figure 3: **Ribbon diagram of the proaerolysin monomer**. Functional domains discussed in the text are labeled.

on the face of domains 1 (Tyr61) and 2 (Trp324) appear to be involved in receptor binding, while other regions of domain 1 may also be involved in dimer stabilization (Mackenzie *et al.*, 1999; Parker *et al.*, 1996). Domains 3 and 4 on the other hand are involved in channel formation (Wilmsen *et al.*, 1992), with domain 4 also containing the activation region (Howard and Buckley 1985; Figure 3).

Secretion

Secretion of proaerolysin requires passage across both the inner and outer membranes. This is accomplished by the type II branch of the General secretory pathway (Pugsley, 1997; Wong and Buckley, 1993). Transport of proaerolysin across the inner membrane is cotranslational and requires a signal sequence that is removed as the protein is transported through the sec system (Howard and Buckley 1985). Once inside the periplasm the protoxin is folded into its native conformation and dimerizes (Hardie *et al.*, 1995). Dimerization appears to be required for proaerolysin secretion, as the large lobe of the toxin which is monomeric, is poorly secreted when expressed by itself (Diep *et al.*, 1998b). Transport of the proaerolysin dimer across the outer membrane involves a group of proteins encoded by the exe genes (Jiang and Howard, 1991 and 1992) and requires energy input in the form of the proton motive force and ATP (Wong and Buckley 1989; Letellier *et al.*, 1997). How energy is coupled to the protein secretion machinery is not known.

Proteolytic activation

Although the activity of aerolysin is greatly enhanced by the presence of membrane receptors, the toxin is able to form channels in any lipid bilayer,

including the membrane of the secreting bacteria. Presumably this is why the toxin is secreted in an inactive form. Once proaerolysin has been released, activation is accomplished by the proteolytic nicking of a region 40 to 50 amino acids from the C-terminus (Howard and Buckley, 1982; van der Goot *et al.*, 1994). This region contains cut sites for a variety of eukaryotic and prokaryotic proteases including trypsin, chymotrypsin, furin, thermolysin, a serine protease, and proteinase K (Abrami *et al.*, 1998; Howard and Buckley, 1982; Garland and Buckley, 1988; van der Goot *et al.*, 1992). Outside of this activation site aerolysin is relatively resistant to proteolysis (Garland and Buckley, 1988).

Depending on the environment proaerolysin is secreted into, a variety of proteases are available for activation. If activation occurs in solution, as may happen in the intestine where trypsin and chymotrypsin are abundant, then active aerolysin will bind to cells. The bacteria also produces at least one protease that is capable of activating proaerolysin (Garland and Buckley, 1988). Alternatively, inactive proaerolysin can bind to the cell and be activated by cell surface proteases such as furin (Abrami *et al.*, 1998).

How proteolytic removal of a small C-terminal peptide allows oligomerization to proceed is not known. Unlike α-toxin of *C. septicum* whose activation peptide appears to stay associated with the toxin following proteolysis, the activation peptide of proaerolysin must move away from the rest of the protein. Engineering a disulfide bridge to prevent the peptide from moving after cleavage results in inactivation of the toxin (van der Goot *et al.*, 1994). Movement of the peptide may be required to destabilize the dimer, which may separate

before oligomerization. Also, peptide movement may trigger a structural change in the molecule or exposure of a hydrophobic patch required for oligomerization. These possibilities are supported by spectroscopic observations, which show there is a change in protein conformation following activation (van der Goot *et al.*, 1994).

Binding

Both proaerolysin and aerolysin are able to bind to target cells. Bernheimer and Avigad proposed that different receptors were present on different cell types and that this accounted for the range of sensitivities observed for various cell types (Bernheimer *et al.*, 1975). The existence of protein receptors was later demonstrated by Howard and Buckley (1985). Subsequent studies on erythrocyte membranes demonstrated that planar lipid bilayers containing erythrocyte proteins are more sensitive to aerolysin than bilayers lacking proteins (Gruber *et al.*, 1994). One of the components of erythrocyte membranes making them more sensitive to aerolysin was identified to be a 47-kDa protein, referred to as the erythrocyte aerolysin receptor (EAR; Cowell *et al.*, 1997). The erythrocyte receptor was purified to homogeneity and N-terminal sequencing revealed it was a novel protein that shares homology with a small family of enzymes involved in ADP-ribosyltransferase reactions (Cowell *et al.*, 1997). However, as results presented in this thesis will show, this enzyme activity appears to have nothing to do with aerolysin activity.

During the purification and characterization of the erythrocyte receptor, a novel method was developed to detect proaerolysin-binding proteins by sandwich

Western blotting. This procedure involves probing blots with proaerolysin, which is then detected by employing a polyclonal anti-aerolysin antibody and an enzyme-linked secondary antibody (Cowell *et al.*, 1997). This is now a routinely used technique for screening different cell types for proaerolysin receptors.

Oligomerization

Following binding, seven aerolysin monomers are thought to oligomerize to form a heptameric oligomer. Based on the crystal structure of the *S. aureus* α-hemolysin channel discussed earlier, and on image analysis of 2-D crystals of the oligomer, molecular modeling suggests that the aerolysin oligomer takes on a mushroom shaped structure that sits in the membrane with the cap flush against the membrane and the stem forming the channel that traverses the bilayer (Wilmsen *et al.*, 1992; Parker *et al.*, 1996; Song *et al.*, 1996). No significant change in secondary structure is observed following the transformation from water soluble dimer to insertion competent oligomer by far UV CD (van der Goot *et al.*, 1994). There is however a change in tertiary structure as revealed by near UV CD (van der Goot *et al.*, 1994). This is consistent with a reorientation of the domains or a change in the positions of aromatic side chains of aerolysin to generate the mushroom shaped heptamer (van der Goot *et al.*, 1994).

How aerolysin is transformed from water soluble dimer to an insertion competent oligomer is unknown. In the dimer, the monomers are orientated in an antiparallel fashion; whereas in the heptameric oligomer, the monomers are thought to be aligned parallel to one another (Parker *et al.*, 1996). To undergo this transformation dimer separation must occur. Receptor binding may trigger dimer

separation although this has yet to be proven. The importance of dimer separation in aerolysin activity was demonstrated by the generation of a covalent dimer formed by genetically engineering a disulfide bridge between the two monomers (Hardie *et al.*, 1995). This covalent dimer was unable to oligomerize (Hardie *et al.*, 1995). Although this evidence supports dimer separation we have not yet determined if a monomeric aerolysin intermediate is generated following binding. The crystal structure and biochemical studies reveal that the toxin exists in solution as a noncovalent dimer (Parker *et al.*, 1994). A recent paper by Fivaz *et al.* suggests that at low toxin concentrations the dimer separates, however this is not consistent with other data from our lab (1999; unpublished). In fact, it appears that the dimer is quite stably held together, as the monomer-monomer interfaces interact *via* a number of bonds including salt bridges, hydrogen bonds and van der Waals interactions (Parker *et al.*, 1994; van der Goot *et al.*, 1993a).

Oligomerization can be promoted or inhibited by a variety of factors. It is a concentration dependent process that can occur at measurable rates in solution at concentrations above 1 uM; however, it can occur at far lower concentrations following receptor binding (Green *et al.*, 1990). Binding serves to concentrate aerolysin, by constraining the molecule to diffusion in 2 dimensions on the cell surface. While binding promotes oligomerization, other factors can inhibit oligomerization. These include raising the pH above 8 or the addition of Zn^{2+} ions (Buckley *et al.*, 1995; Wilmsen *et al.*, 1990). It was determined that the elevated pH inhibited oligomerization by resulting in deprotonation of a critical histidine residue required for channel formation (Buckley *et al.*, 1995). The addition of

Zn^{2+} ions not only resulted in inhibition of oligomerization, but also resulted in the closure of channels, apparently as a result of the binding of three Zn^{2+} ions within the lumen of the channel (Wilmsen *et al.*, 1990).

Once formed, the aerolysin oligomer is extremely stable. It is resistant to detergent, weak acid or base, urea and elevated temperature (Buckley, 1999; Lesieur *et al.*, 1999). However, the structure is noncovalent since it can be dissociated by formic acid (Moniatte *et al.*, 1997). The stability of the oligomer has allowed characterization by SDS PAGE and MALDI-TOF mass spectroscopy (Moniatte *et al.*, 1996).

Channel formation

Once inserted into the plasma membrane of target cells, the oligomer becomes a transmembrane channel that spans the bilayer (Parker *et al.*, 1996). The functional properties of this channel have been studied using planar lipid bilayers (Wilmsen *et al.*, 1990). The channel has a conductance of 420 pS and it remains open between –70 and +70 mV (Wilmsen *et al.*, 1990). It is slightly anion selective and as mentioned above, the addition of Zn^{2+} to preformed channels can cause them to close in a voltage dependent manner (Wilmsen *et al.*, 1990).

Based on electron microscopy images of 2-dimensional oligomer arrays, a 3-dimensional model of the oligomer was proposed (Wilmsen *et al.*, 1992). According to this model, the aerolysin channel would have an internal diameter of approximately 1.7 nm. In agreement are calculations based on scanning microphotolysis suggesting that the pore is 1.9 to 2.3 nm in diameter (Tschodrich-Rotter *et al.*, 1996). Still other studies measuring the release of molecular mass

markers from erythrocytes ghosts revealed that the largest molecule that can pass through the aerolysin channel is 3500 Da, also suggesting that the channel is 1.5 to 3 nm in diameter (Howard and Buckley 1982).

Summary

The focus of this dissertation is the binding of aerolysin to cell surface receptors and the influence of binding on activity. The interaction of aerolysin and other toxins with their receptors will be discussed below. In addition, since I will show that GPI-anchored proteins (GPI-APs) are the receptors for aerolysin, their structure and function will be discussed along with their ability to localize in raft domains.

Toxin-Receptor Interactions

Before a toxin can exert an affect on a target cell, it must bind to the surface of that cell. To accomplish this, toxins use a variety of surface molecules to which they bind with high affinity. Before considering specific examples of toxin-receptor pairings, it is first necessary to address the types of interactions involved in toxin-receptor binding.

Forces involved in toxin-receptor interactions

Any combination of ionic bonds, hydrogen bonds, van der Waals attractions and hydrophobic forces may be involved in the binding of a toxin to a receptor. The length of these noncovalent interactions ranges from 0.25 nm for ionic bonds, 0.3 nm for hydrogen bonds and 0.35 nm for van der Waals interactions. Thus, the toxin and receptor must be in close contact before any

bonds are formed (Alberts *et al.*, 1994). Once formed, these bonds are 30 to 300X weaker than covalent bonds, and are only slightly stronger than the energy released following a thermal collision (Alberts *et al.*, 1994). Therefore, to prevent a toxin-receptor interaction from being pulled apart by thermal motion, several noncovalent bonds must form (Alberts *et al.*, 1994).

Binding affinity

When a toxin combines with a receptor in such a way that a number of energetically favorable bonds can form between the two molecules, binding occurs. Weak interactions are quickly pulled apart by thermal motion whereas strong interactions are maintained for longer periods of time (Alberts *et al.*, 1994). The strength of these interactions is measured by the equilibrium constant (Alberts *et al.*, 1994). As with typical receptor-ligand interactions, when a toxin (T) and receptor (R) bind they will form a toxin-receptor (TR) complex. This interaction will proceed until an equilibrium point is reached where the velocity of formation and dissociation are equal (Alberts *et al.*, 1994).

Dissociation rate [TR] → [T][R] = dissociation rate x [TR]

Association rate [T][R] → [TR] = association rate x [T][R]

At equilibrium the rate of association and dissociation are equal therefore, we are left with an equation for the equilibrium constant:

$$\frac{[TR]}{[T][R]} = K_A = \text{Equilibrium constant}$$

The concentrations of free toxin, receptor, and toxin-receptor complex can be used to determine the equilibrium or affinity constant. The higher the

concentration of toxin-receptor complex, the larger the affinity constant, and therefore the stronger the binding of a toxin to its receptor (Hornak *et al.*, 1999). The reciprocal of the K_A is the dissociation constant (K_D) so the smaller the K_D the higher the affinity of two molecules for one another (Alberts *et al.*, 1994).

With high affinity binding, several noncovalent bonds form, and there is a release of free energy (Hornak *et al.*, 1999). If there is more free energy released upon toxin-receptor binding than the energy in the free states of interacting molecules, then the equilibrium will shift in favour of toxin-receptor complex formation (Alberts et al., 1994). For example, if the aerolysin dimer does dissociate and more bonds form between a GPI-anchored receptor and aerolysin than were formed between the two monomers in the dimer, then receptor binding would favour dimer dissociation.

Types of toxin-receptor interactions

Most toxins are required to bind to, be transported across, or form a membrane spanning channel in the plasma membrane. The plasma membrane of cells like erythrocytes is thought to be covered in a glycocalyx that is generated by the carbohydrate side chains of lipids and proteins that are embedded or anchored in the membrane (Wall *et al.*, 1995). It is therefore not surprising that toxins often bind to glycoproteins or glycolipids on the plasma membrane (Karlsson, 1995). The forces involved in these interactions are often protein-carbohydrate or protein-protein interactions.

Protein-carbohydrate interactions

Each carbohydrate binding domain on proteins typically interacts with three to four monosaccharides (Evans *et al.*, 1999). These interactions involve weak hydrophobic attractions and hydrogen bonds (Evans *et al.*, 1999). Hydrophobic interactions occur between the rings of aromatic amino acid side chains and the hydrophobic sugar rings found on carbohydrates. These two types of planar rings stack against each other allowing for the formation of extensive hydrophobic contacts (Evans *et al.*, 1999). Hydrogen bond networks on the other hand form between polar amino acid side chains and hydroxyl groups and other polar residues on carbohydrate chains (Evans *et al.*, 1999). Hydrophobic interactions and hydrogen bonds are relatively weak and since the protein binding site only interacts with three to four monosaccharides, the binding affinity of each site is usually quite low (K_D 10^{-3} to 10^{-5} M; Evans *et al.*, 1999; MacKenzie *et al.*, 1996). To compensate for this low binding affinity, carbohydrate binding proteins often contain several binding subunits (Mammen *et al.*, 1998).

Protein-protein interactions

Protein-protein interactions are typically of higher affinity (K_D 10^{-9} M) than protein-carbohydrate interactions (Evans *et al.*, 1999). This is because a number of noncovalent bonds can form between amino acid side chains of two proteins, including the strongest of noncovalent bonds, the ionic bond between basic and acidic residues on opposing surfaces. In addition hydrogen bonds and van der Waals interactions can occur between side chains of amino acids at the protein-protein interface.

Method for studying toxin-receptor interactions

Surface plasmon resonance is a novel technique that has been used to study toxin-receptor interactions in real time. This is accomplished by monitoring changes in the resonance angle of light impinging on a gold surface (measured in resonance units), caused by changes in the refractive index of a second surface that is located approximately 300 nm below the gold surface (Lakey and Raggett, 1998). The second surface contains immobilized receptor. As soluble toxin is passed over the receptor surface its refractive index changes as a result of toxin-receptor complex formation (Lakey and Raggett, 1998). There is a linear correlation between changes in refractive index and changes in resonance units (Phizichky and Fields, 1995). Since all proteins have approximately the same refractive index, changes in resonance units can be taken as an indication of protein concentration (Phizichky and Fields, 1995). The changes in protein concentration can then be used to determine the amount of complex formed. Since these changes are monitored over time, the rate of association and dissociation can also be determined (Lakey and Raggett, 1998). This technique has been used to study the interaction of aerolysin with GPI-APs (MacKenzie *et al.*, 1999).

Toxin Receptors

A small number of specific receptors have been identified for individual bacterial toxins. Representative examples of these receptors and some general properties of the toxin binding domains will be considered here.

Binding domain

Since carbohydrates decorate the surface of cells, they make attractive targets. Many toxins are known to recognize specific carbohydrate determinants. Analysis of the structures of the binding domains of these proteins has revealed two folds that are involved in carbohydrate recognition. These are the oligosaccharide binding (OB) fold, and the aerolysin-pertussis toxin (APT) domain (Merritt and Hol, 1995; Rossjohn *et al.*, 1997a). The OB fold is found in a number of toxins with a hexameric (AB_5) architecture, including CT and PT, while the APT domain is so far restricted to the small lobe of aerolysin and the two subunits of PT (S2 and S3) although this domain is similar to the carbohydrate-recognition domain (CRD) found in a large number of eukaryotic proteins (Merritt and Hol, 1995; Murzin, 1993; Rossjohn *et al.*, 1997a). The OB fold seems to be involved in binding to carbohydrate determinants on gangliosides, whereas the APT domain has been suggested to be involved in the recognition of carbohydrate determinants on glycoproteins (Merritt and Hol, 1995; Rossjohn *et al.*, 1997).

Ganglioside Receptors

One group of toxins that appear to almost exclusively bind gangliosides are the hexameric enzymatic toxins. The B subunits of all AB_5 enzymatic toxins studied to date contain an OB fold. The crystal structure of these folds reveals that each consists of two β-sheets that form a β-barrel capped by a long α helix which binds a carbohydrate determinant (Stein *et al.*, 1994). This fold is used by each B subunit on CT to bind the terminal oligosaccharide portion

(GalNAcβ1(NeuAcα2→3)4Gal) of G_{M1}, (Scott *et al.*, 1996). The affinity of G_{M1} binding to one B subunit is low; however, because binding is pentavalent the avidity constant is high (K_D 7x 10^{-10} M; MacKenzie *et al.*, 1996).

Unlike CT, PT is an enzymatic AB_5 toxin whose pentameric receptor binding domain contains non-identical B subunits. Only two of these B subunits, which share 70% sequence identity, are thought to be involved in carbohydrate binding (Rossjohn *et al.*, 1997). This may account for the lower affinity binding of PT to ganglioside GD_{1a} than is observed for pentameric binding of CT to G_{M1} (Hausman and Burns, 1993; Scott *et al.*, 1996).

Glycoprotein Receptors

The involvement of the OB folds in carbohydrate recognition by PT is supported by the fact that the crystal structure of PT with a bound ligand revealed a sialic acid binding site in the OB fold (Stein *et al.*, 1994). However, this site was located near the catalytic site away from the base of the pentamer which is believed to interact with the membrane. Alternatively, since the carbohydrate side chains of many glycoproteins contain terminal sialic acid residues, PT may use this site to bind to a glycoprotein receptor (Stein *et al.*, 1994). A variety of glycoproteins that bind pertussis toxin have been identified, but their role in intoxication has yet to be demonstrated (Armstrong *et al.*, 1994; Clark and Armstrong, 1990; Rogers *et al.*, 1990).

In addition to the OB fold, the two subunits involved in receptor binding also contain an APT domain (Rossjohn *et al.*, 1997a). The fact that the APT domain is only found in the two subunits involved in binding suggests this

domain may interact with a surface receptor. In addition, this domain is located near the base of the pentamer which as mentioned above is believed to interact with the membrane. In spite of this, the role of the APT domain in binding to a specific receptor is yet to be demonstrated.

Like PT, the small lobe of aerolysin contains an APT domain (Rossjohn *et al.*, 1997a). This domain is involved in binding to GPI-anchored aerolysin receptors (MacKenzie *et al.*, 1999; unpublished). In addition, a second region on the top of domain two of aerolysin contains aromatic amino acids (Y162 and W324), which are characteristic of carbohydrate binding domains, and have been shown to be involved in binding (Vjas, 1991; MacKenzie *et al.*, 1999). Corresponding aromatic residues (Y93 and Y262) are found on *C. septicum* alpha-toxin (Ballard *et al.*, 1995). Interestingly, these two toxins both use glycosylphosphatidylinositol (GPI)-anchored protein receptors, suggesting that the aromatic residues may be involved in GPI-anchor recognition (Nelson *et al.*, 1997; Diep *et al.*, 1998a; Gordon *et al.*, 1999).

Another pore-forming toxin using a GPI-AP receptor is CryIAc from *Bacillus thuringiensis* (Knight *et al.*, 1995). This toxin binds to aminopeptidase N, a 120-kDa GPI-AP found on brush border cells lining the insect larvae midgut (deMaagd *et al.*, 1999). Unlike aerolysin and alpha-toxin however, CryIAc only seems to use one GPI-anchored receptor and it is not yet determined if it must oligomerize to become active.

Glycosylphosphatidyl-inositol anchored proteins:

Structure

Historically, integral cell surface proteins were thought to be attached to the plasma membrane by transmembrane peptide segments. The first hint of the existence of another protein anchoring mechanism came in the late 1970's with the simultaneous finding from the labs of Ikezawa and Low that alkaline phosphatase could be released from mammalian cell surfaces by the bacterial enzyme phosphatidylinositiol-specific phospholipase C (PI-PLC; Ikezawa *et al.*, 1976; Low and Finean, 1977). The suggestion by both Low and Ikezawa was that alkaline phosphatase interacts with the plasma membrane via a novel mechanism utilizing the phospholipid phosphatidylinositol (PI). Following this initial finding, several other groups identified other PI-anchored proteins on various cell types. Analysis of the anchor structure of trypanosomal variant surface glycoprotein (VSG), a representative PI-anchored protein, revealed that the anchor not only contained PI, but in addition a glycan core (Ferguson *et al.*, 1985). The composition of this anchor resulted in this class of proteins being named glycosylphosphatidylinositol (GPI)-anchored proteins (Ferguson *et al.*, 1988; Low *et al.*, 1987). Since the initial discoveries, over 200 GPI-APs have been identified (Treumann, *et al.*, 1998; McConville and Ferguson, 1993; Ferguson, 1999) and the anchor structures of at least 25 of them have been solved (Brewis *et al.*, 1995; Ferguson *et al.*, 1985; Misume *et al.*, 1990; Roberts *et al.*, 1987; Figure 4). They all are comprised of a phosphoethanolamine residue attached to a glycan core containing at least three mannose residues and a glucosamine, in turn attached to

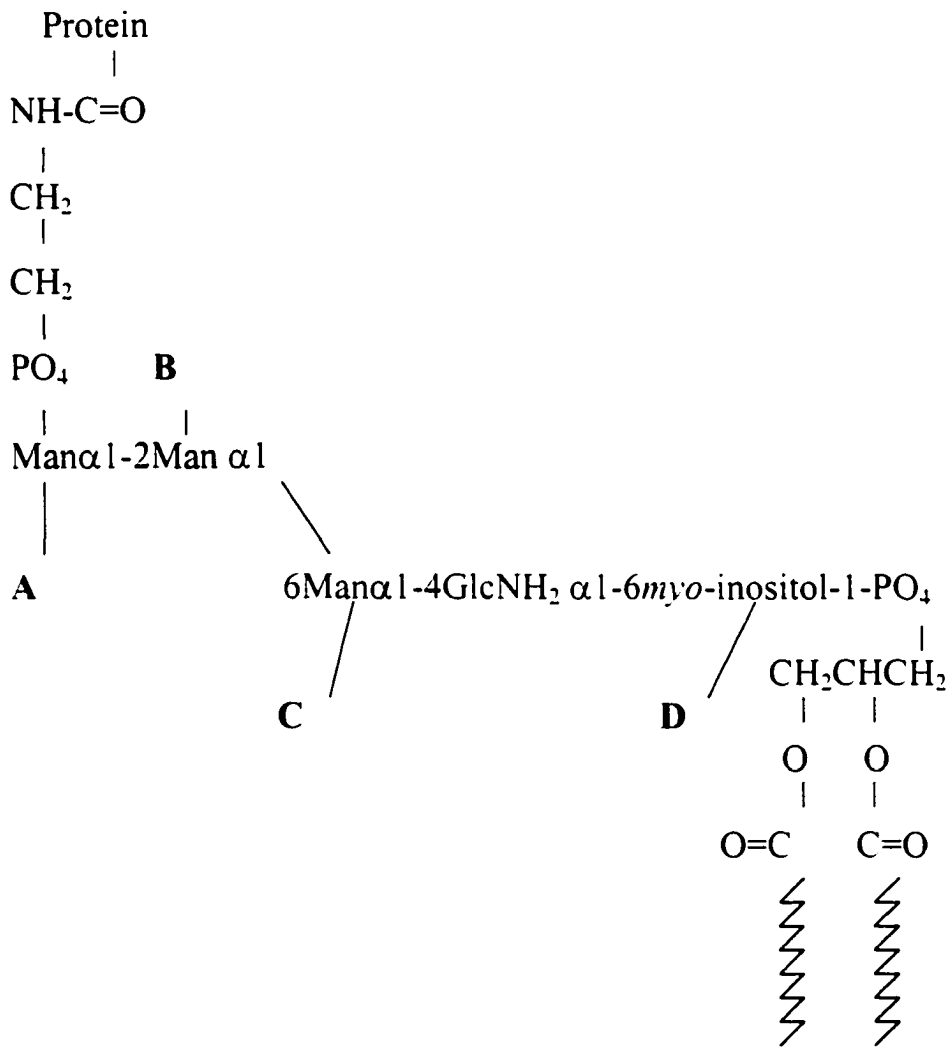


Figure 4: **Conserved core structure of a GPI-anchor**. Site of addition of sugar or phosphoethanolamine residues (A, B, and C) or an additional acyl chain (D) are indicated.

an inositol ring that is phosphodiester linked to a molecule of diacylglycerol. It is the diacylglycerol that anchors the protein in the membrane (reviewed in McConville and Ferguson, 1993).

Although all species share a common core GPI-anchor, this core is variably modified in different species and cell types (Englund, 1993; Paturiaux-Hanocq *et al.*, 1997; Treumann *et al.*, 1998). Structural heterogeneity observed between GPI-anchors is the result of modifications at three sites on the core (Figure 4). First, the glycan core can be variably decorated with mannose or galactose residues and it may contain an additional phosphoethanolamine residue. Second, the inositol ring of some GPI-anchors may be acylated (for example, human erythrocyte GPI-APs; Deeg *et al.*, 1992). Finally, different acyl chains can be added to the glycerol backbone (Brewis *et al.*, 1995; Treumann *et al.*, 1998). Anchor modification may be protein, cell, tissue or species specific. The evidence available suggests that it may be cell or tissue specific, because different anchor structures can occur in a given species. For example, an additional ethanolamine residue modifies the core anchor structure for human placental alkaline phosphatase, whereas additional sugar and palmitate residues are added to the anchor of CD52 from spleen (Redman *et al.*, 1994; Treumann *et al.*, 1995). The only example of two different GPI-APs analyzed from the same cell are human erythrocyte actetylcholinesterase and CD59 (Deeg *et al.*, 1992; Roberts *et al.*, 1987; Rudd *et al.*, 1997). The anchor structures of these two proteins were identical, apart from a small proportion (9 %) of the CD59 anchors which had an extra GalNac (Rudd *et al.*, 1997). Although this suggests that anchors on a given

cell are quite similar, the ability of a given cell type to use more than one different anchor structure was also demonstrated by the variable modification of the anchor of porcine kidney membrane dipeptidase (Brewis *et al.*, 1995).

Biosynthesis

Addition of a GPI-anchor to a protein involves two separate processes; the biosynthesis of the anchor, and the processing of the nascent protein and concurrent attachment of the anchor (Udenfriend and Kodukula, 1995; Takeda and Kinoshita, 1995). The anchor is synthesized on the cytoplasmic side of the endoplasmic reticulum (ER; Vidugiriene and Menon, 1993). The first step involves the transfer of GlcNAc to phosphatidylinositol anchored in the outer leaflet of the ER. This transfer is accomplished by the GlcNAc transferase complex, which is composed of several proteins including phosphatidylinositol glycan A (PIG-A; Watanabe *et al.*,.1996). A defect in the *pig-A* gene occurs in the disease paroxysmal nocturnal hemoglobinuria (PNH), which is characterized by a lack of expression of GPI-APs on hematopoietic cells (Rosti, 2000). Fortunately, this is a rare disease affecting an estimated 1 to 10 people/million (Rosti, 2000). Most individuals express PIG-A allowing for subsequent progression through the anchor biosynthesis pathway. The next step in anchor biosynthesis involves the deacetylation of GlcNAc to produce GlcN (Vidugiriene and Menon, 1993). Depending on the cell the inositol ring may then be acylated. This is followed by the sequential addition of three mannose residues and a phosphoethanolamine (Hong *et al.*, 1999). At some point along this pathway the anchor is flipped to the luminal side of the ER, however the point at which this occurs is unknown

(Ferguson, 1999). Once the complete GPI-anchor is facing the lumen of the ER, it is ready to be transferred to the nascent protein, which is anchored to the ER membrane by a 20 to 30 amino acid hydrophobic C-terminal signal sequence (Coyne *et al.*, 1993). A transamidase complex, also embedded in the ER membrane, cleaves this signal sequence and transfers the anchor *en bloc* to the C-terminus of the awaiting protein (Ramalingam *et al.*, 1996).

Properties

The discovery of this unique form of attachment to the cell membrane has generated a great deal of interest in what functional attribute it could provide the protein. One possibility is that GPI-anchors provide the protein with a high degree of lateral mobility on the surface of the cell compared with transmembrane proteins. In fact, the diffusion coefficient for some GPI-APs ($D \sim 10^{-9}$ cm^2/sec) approach that of a lipid molecule ($D \sim 10^{-7}$ to 10^{-9} cm^2/sec; Ishihara *et al.*, 1987; Kooyman *et al.*, 1995; Cullus *et al.*, 1996). In comparison, membrane spanning proteins have low diffusion coefficients ($D \sim 10^{-10}$ to 10^{-13} cm^2/sec) due to interactions of the cytoplasmic domains with cytoskeletal proteins and to the increased energy involved in moving the transmembrane domains through the membrane (Cowan *et al.*, 1987; Thomas *et al.*, 1990). In spite of this difference, there are also examples of transfected GPI-anchored hybrid proteins that have diffusion coefficients comparable to transmembrane proteins (Jacobson *et al.*, 1997; Simson *et al.*, 1998; Zhang *et al.*, 1991), indicating that other factors, such as the interaction of the extracellular domain of the protein with other cell surface components, may also influence lateral mobility (Zhang *et al.*, 1991).

The PI-PLC cleavage site is certainly a unique feature of GPI-APs. Cleavage may allow the cell to rapidly alter the architecture of the cell surface with endogenous PI-PLC (Lehto and Sharom, 1998; Taguchi *et al.*, 1985). To comprehend what impact this could have for the cell we must first look at what functional roles GPI-APs have on the cell surface.

Functions

GPI-APs have a diverse range of functions. They may be cell surface receptors (Thy-1), cell adhesion molecules (contactin), enzymes (placental alkaline phosphatase; PLAP), or lymphoid antigens (Kroczek *et al.*, 1986; Lemansky *et al.*, 1990; Redman *et al.*, 1994; Reid *et al.*, 1994). Others are complement regulatory proteins. For example, DAF protects the body from homologous complement and CAMPATH-1 serves as a complement target (reviewed in Rosti, 2000). The importance of GPI-APs in development and/or cell to cell signaling is demonstrated by the fact that knockout mutations in mice are lethal, whereas an individual cell type with a similar GPI-knockout can survive well in culture (Sugiyama *et al.*, 1991; Tarutani *et al.*, 1997). This suggests a critical role for GPI-APs in development or cell to cell signaling, and a nonessential role on individual cells.

While GPI-APs are found distributed on the entire surface of the plasma membrane, they are said to associate with specialized domains on the plasma membrane called lipid rafts (Benting *et al.*, 1999; Brown and London, 2000; Nosjean *et al.*, 1997; Simons and Ikonen, 1997). The functional significance of

GPI-APs has recently been attributed to their association with signaling molecules in lipid rafts (Harder and Simons, 1999; Lipardi *et al.*, 2000; Kramer *et al.*, 1999).

Lipid Rafts

Recently it was suggested that rafts function as concentration platforms that facilitate channel formation by aerolysin (Abrami and van der Goot, 1999). Results will be presented here describing the association of aerolysin with GPI-APs in rafts and the lack of functional significance to this association.

Structure and components

The nature of the lipids comprising the lipid bilayer allows for phase separations to occur within the membrane (Rietveld and Simons, 1998). Phospholipids and sphingolipids generally exist in lipid bilayers in one of two phases, the fluid liquid crystalline phase (l_c) where acyl chains are fluid and disordered, and the solid gel like phase where acyl chains are ordered and tightly packed together (Brown and London 1998b). For a given lipid, the transition between these two phases occurs at a characteristic transition temperature (T_m). Glycerophospholipids contain acyl chains (16 to 22 carbons) that have 1 to 1.5 cis unsaturated bonds; because of this they have a low T_m (Barenholz *et al.*, 1980). In comparison, sphingolipids have relatively long acyl chains (20 to 26 carbons) that typically contain only 0.1 to 0.35 cis unsaturated fatty acids and (Barenholz *et al.*, 1980), because of this they can tightly pack together and have a higher T_m (Brown and London, 1998a). When these two lipid species are mixed in artificial lipid bilayers, a phase separation can occur, and l_c and gel phase domains will coexist

in the bilayer (Brown and London, 1998b). When cholesterol is also present a unique phase called the liquid ordered (l_o) phase may exist, (Brown and London, 2000; Schroeder *et al.*, 1998). The l_o phase, which contains cholesterol and high T_m lipids, has characteristics intermediate between l_c and gel phases (Brown and London, 2000). As in the l_c phase, lateral motion of lipids is rapid, however as in the gel phase, the acyl chains of saturated lipids are tightly packed and extended (Brown and London, 1998b). These liquid ordered domains are models for lipid rafts in cell membranes (Brown and London, 2000).

Forces involved in maintaining raft domains

One of the main factors thought to stabilize raft domains is the tight packing of saturated acyl chains with cholesterol (Brown and London, 2000). Cholesterol fills in the gaps that are created between acyl chains because of the head groups of sphingolipids occupying a larger surface area than their saturated acyl chains (Harder and Simons, 1997). The cholesterol also stabilizes the structure by interacting along its entire length with the hydrophobic acyl chains (Ilangumaran and Hoessli, 1998). Other cohesive forces within raft domains may include hydrogen bonds between the sugar head groups of glycosphingolipids and van der Waals interactions between the sphingosine backbones of the sphingolipids (Harder and Simons, 1997). These cohesive forces come together to generate a stable raft domain floating in a sea of fluid glycerolipids (Harder and Simons, 1997; Rietveld and Simons, 1998).

Evidence for the Raft Model

The initial evidence for the existence of rafts was based on their insolubility in cold Triton X-100 (Fra et al., 1994; Schroeder *et al.*, 1994). This insolubility is a result of the combination of the cohesive forces mentioned above and is a distinguishing property of raft domains. For this reason rafts are also know as detergent insoluble domains or detergent resistant membranes. These detergent insoluble domains can be isolated by sucrose density gradient centrifugation, or by high-speed centrifugation of Triton X-100 extracted cells (Fra et al., 1994).

Evidence of the existence of lipid rafts *in vivo* has come from studies of the distributions of the GPI-AP Thy-1, and the glycosphingolipid G_{M1} on the plasma membrane of fibroblasts by single particle tracking (Sheets *et al.*, 1997). This technique allows the movement of individual molecules labeled with fluorescent particles or colloidal gold to be monitored over time with nanometer precision by video-enhanced brightfield microscopy. Analysis of Thy-1 and G_{M1} demonstrated both were transiently confined to regions averaging 260 to 370 nm in diameter (Sheets *et al.*, 1997). Reducing glycosphingolipid expression in the cell by approximately 40% with a glucosylceramide inhibitor decreased the size of the confinement zone 1.5 fold (Sheets *et al.*, 1997). Thus, these confinement zones may correspond to lipid rafts in biological membranes (Sheets *et al.*, 1997).

In addition to single particle tracking, fluorescence resonance energy transfer (FRET) microscopy of a fluorescent folate analog was used to compare the distributions of transfected GPI-anchored and transmembrane folate receptor

on the cell surface (Varma and Mayor, 1998). When fluorescence resonance energy transfer occurs between the fluorescent folate analogs, there is a simultaneous loss in polarization (increase in anisotropy) because energy transfer causes the emission to become depolarized (Varma and Mayor, 1998). In this case FRET was used in a novel way to look at concentration dependent depolarization on unfixed cells (Varma and Mayor, 1998). If one decreases the density of a protein that is randomly distributed on the cell surface, there will be a corresponding decrease in anisotropy as the distance between molecules is greater and energy transfer between the two molecules can no longer be as efficient (Varma and Mayor, 1998). However, if proteins are organized in submicron sized domains, and one decreases the density of fluorophore, there is no change in anisotropy as energy transfer can still occur just as well as before, since the receptors, being clustered in the submicron sized domains, are still close enough together for energy transfer (Varma and Mayor, 1998). GPI-anchored folate receptor showed no decrease in anisotropy following dilution of the fluorophore on the cell surface, while the transmembrane form of the folate receptor did undergo a decrease in anisotropy following a decrease in fluorophore density (Varma and Mayor, 1998). This is evidence that GPI-APs are localized in submicorn sized domains while transmembrane proteins are not. Based on these studies, submicron raft domains were determined to be approximately 70 nm in diameter and to contain approximately 50 molecules of GPI-AP (Varma and Major, 1998). These studies support the view that raft microdomains exist on the plasma membrane.

Conventional FRET was also used on fixed cells to look at the distribution of another GPI-AP, 5' nucleotidase. In contrast to the experiment just described, which used one fluorophore, here the distribution of 5' nucleotidase was examined by looking at energy transfer between the two fluorophores Cy3 and Cy5 conjugated to antibodies against 5' nucleotidase (Kenworthy *et al.*, 1998). The results obtained in this study did not show clustering in submicron sized domains suggesting a random distribution of GPI-anchored 5' nucleotidase on the cell surface.

The discrepancies in these two FRET studies of GPI-AP localization in rafts may be reconciled by a recent third set of FRET studies and a critical analysis of the existing data (Kenworthy *et al.*, 2000). Here again conventional FRET was used and as above these authors concluded that most of the GPI-anchored folate receptor, CD59 and 5' nucleotidase was not clustered in raft domains. These authors propose that raft domains may be even smaller than 70 nm and are therefore too small and/or too few in number to detect (Kenworthy *et al.*, 2000). In addition, these authors cite personal communication with Mayor, the coauthor of the nonconventional FRET studies, which suggests that the calculations based on the distribution of fluorescent folate analog are consistent with the enrichment of only a fraction of the folate receptor in clusters with the remainder being randomly distributed at low density outside of the raft domain (Kenworthy *et al.*, 2000; Varma and Mayor, 1998). Despite intense effort the exact structure of raft domains remains unclear and researchers are still trying to determine what fraction of GPI-anchored proteins are localized to these domains.

Microdomains of GPI-APs were revealed on living cells using chemical crosslinking (Friedrichson and Kurzchalia, 1998). If GPI-APs are localized to submicron sized domains then it should be possible to chemically crosslink them. Madin-Darby canine kidney (MDCK) cells expressing GPI-anchored decay accelerating factor (DAF) were cross-linked with the membrane impermeable agent bis (sulphosuccinimidyl)-suberate (BS3). Analysis of cells by SDS-PAGE after cross-linking revealed a range of crosslinked proteins corresponding to dimers of DAF and aggregates of up to 15 molecules of DAF. To confirm that these were indeed aggregates of DAF, the cleavable BS3 analog 3,3'-dithiobis-(sulphosuccinimidylpropionate) (DTSSP) was used followed by two-dimensional electrophoresis. The first dimension revealed a pattern similar to that obtained with BS3. Running the gel in the second dimension under reducing conditions resulted in the crosslinker DTSSP being cleaved, releasing monomeric subunits of 23-kDa, corresponding to DAF. The fact that the spacer arm of BS3 is only 1.14 nm long indicates that these GPI-APs are indeed in close proximity on the surface of the plasma membrane. To confirm that these proteins were localized in raft domains, cells were treated with the cholesterol sequestering agent methyl-β-cyclodextrin, which extracts cholesterol from plasma membranes thereby disrupting lipid rafts. Following raft disruption, cells were again treated with the crosslinker BS3 and the cells were checked for aggregates. Once rafts were disrupted, DAF molecules were no longer crosslinked, indicating that the DAF, which had been in raft domains, was now randomly distributed on the surface of

the cell (Friedrichson and Kurzchalia, 1998). This suggests that raft domains containing GPI-anchored DAF do exist on the cell surface.

Function

Interestingly, several proteins containing other kinds of lipid anchors are also found in rafts (Benting *et al.*, 1999). It is believed that their saturated fatty acyl chains favour an association with the saturated lipids in raft domains (Schroeder *et al.*, 1994). These acylated proteins include signaling molecules, which led to the suggestion that rafts function as signaling centers. There have also been reports of GPI-APs being clustered into rafts and triggering intracellular signaling (Brown, 1993; Horejsi *et al.*, 1998; Moran *et al.*, 1998). The association or clustering of proteins in lipid rafts could affect protein function by favouring the interaction of GPI-APs with signaling molecules found in the raft domains (Brown and London, 2000; Stunlig *et al.*, 1998).

The observation that rafts are found on apical and not basolateral surfaces of epithelial cells has led to the proposal that they function as platforms that transport specific membrane proteins to apical domains in epithelial cells (Lipardi *et al.*, 2000). This model proposes that specific membrane proteins, sphingomyelin and cholesterol associate in the *trans*-Golgi network forming rafts that are then transported to the plasma membrane (Mukherjee *et al.*, 1999).

Purpose of this dissertation

The purpose of this dissertation was to investigate the ability of aerolysin to interact with GPI-APs. In addition, the consequences of this interaction were examined to provide new insight into the effect of aerolysin on T lymphomas.

MATERIALS AND METHODS

Materials

Alexa 488 labeling kits, carboxyfluorescein, Fluo3-AM, and PoPro-1 were purchased from Molecular Probes. The detergents N-laurylsarcosine, t-Octylphenoxypolyethoxyethanol (Triton X-100) and polyoxyethylene-(8)-isooctylphenyl ether (Triton X-114) were purchased from Sigma while octylglucoside was from Bachem. Unless otherwise indicated all salts and buffer reagents were from BDH. Tris-(hydroxymethyl)aminomethane was from ACP. Acrylamide and N,N'-methylene-bisacrylamide for polyacrylamide gels were from BDH and BIORAD respectively. Temed, ammonium persulfate (APS), and HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid]) were from Sigma. Prestained low range molecule mass standards, nitrocellulose (0.2 μm), polyvinylidene difluoride (PVDF), agar for agarose electrophoresis and the one kilobase DNA ladder were all from BIORAD. Sodium dodecyl sulfate (SDS) and polyoxyethylene(20) sorbitan monolaurate (Tween-20) were from Fisher Scientific. Skim milk powder was from DIFCO. Reagents for enhanced chemiluminescence were supplied by NEN Life Science.

Liver phosphatidylcholine (PC), phosphatidylethanolamine (PE) and brain sphingomyelin (SM) were obtained from Avanti Polar lipids. Cholesterol, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, bovine serum albumin (BSA), trypsin, and a crude preparation of human placental alkaline phosphatase were purchased from Sigma. Purified Thy-1 from rat thymus was obtained from Dr. R. McMaster (University of British Columbia (UBC)).

Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), L-glutamine, mercaptoethanol and penicillin/streptomycin for tissue culture were supplied by Gibco. Tris buffered phenol was also supplied by Gibco. Chloroform was from ACP, acetone was from BDH and methanol and isopropanol were from Caledon. Fetal clone I serum for tissue culture was obtained from HyClone. Phosphatidylinositol-specific phospholipase C (PI-PLC) was from Boehringer Mannheim. Peptide-N-glycosidase F was from Oxford Glycosystems. Methyl-β-cyclodextrin, DABCO (1,4-diazobicyclo [2,2,2] octane), and PMS (phenazine methosulfate) were from Sigma. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was from Promega. Capase-3 assay was from Clontech.

Buffers

Sample buffer for SDS PAGE contained 8 % SDS, 20 % β-mercaptoethanol, 40 % glycerol, 108 mM H_2SO_4, 220 mM Tris-HCl pH 6.1 with bromophenol blue. Prior to use sample buffer was either diluted 1:4 in liquid samples or diluted 1:4 in dH_2O and then added to dried pellets or samples. Sample buffer for agarose electrophoresis contained 40 % sucrose, 60 mM EDTA, 0.25 % bromophenol blue and was diluted 1:6 in sample for use. TAE buffer for agarose electrophoresis contained 40 mM Tris acetate, 1 mM EDTA. Phosphate buffered saline (PBS) contained 10 mM NaH_2PO_4, 0.15 M NaCl at pH 7.4. PBS-Tween used in blotting contained 0.5% Tween-20. HEPES buffered saline (HBS) contained 20 mM HEPES, 0.15 M NaCl at pH 7.4.

Toxin production

Proaerolysin variants with single amino acid mutations Tyr Y221 to Gly (Y221G), Thr 253 to Cys (T253C) Ala 300 to Cys (A300C), and with double substitutions Thr 253 to Cys/ Ala 300 to Cys (T253C/A300C) were constructed by site directed mutagenesis and then cloned into pMMB66HE for expression in a protease-deficient strain of *A. salmonicida* (Buckley, 1990; Buckley *et al*, 1995; Green *et al.*, 1990). Wild-type proaerolysin and proaerolysin variants were constructed, produced, and purified by Tracy Lawrence as described previously (Buckley *et al*, 1995; Green *et al.*, 1990; Buckley, 1990).

Fluorescently labeled aerolysin

The purified variants and wt proaerolysin were labeled by Dr. J.T. Buckley with the fluorescent probe Alexa 488 (Molecular Probes), according to the directions provided by the manufacturer.

Antibodies

Polyclonal anti-aerolysin antibody was previously prepared by immunizing a rabbit with denatured aerolysin and collecting the serum. Polyclonal anti-CRD antibody against GPI-anchors was obtained from Oxford Glycosystems. Monoclonal anti-GP63 was obtained from Dr. R. McMaster at UBC. Anti-rabbit horseradish peroxidase and anti-mouse horseradish peroxidase were obtained from Cedarlane.

Equipment

Tissue culture cells were maintained in a Forma Scientific Water Jacketed CO_2 incubator. Optical density was measured using a Varian Cary I spcectrofluorimeter. A QM-1 Photon Technology Instruments spectrofluorimeter was used for fluorometry. Flow cytometry was performed on a FACSCalibur using Cell Quest software (Becton Dickinson). Confocal microscopy was performed on a Zeiss Confocal Laser Scanning Microscope. Liposome sizing was preformed using a Nicomp submicron particle sizer. Columns, pumps and fraction collectors were all from Pharmacia. Gel and blot imaging was performed on an AlphaInnotech ChemiImager 4000 gel documentation system.

Cell lines

Murine T lymphoma cell lines EL4, and AKR1 and their derivatives, EL4 (Thy-1-f), and AKR1 (Thy-1-d), were generously provided by Dr. R. Hyman (Salk Institute). Samples of CHO cells or *Leishmania major* expressing GP63 were obtained from Dr. R. McMaster (UBC).

Tissues

Animal tissues and rat blood were obtained from animals being sacrificed by Animal Care at the University of Victoria, Victoria, B.C.. Whole bovine blood was obtained from KPN. Outdated human blood was obtained from Transfusion Services at the Royal Jubilee Hospital, Victoria, B.C..

Methods

Cell culture

All lymphoma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v bovine fetal clone I serum, 2 mM L-glutamine, 55 μM mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37 °C.

Sample preparation for SDS PAGE

Cell lysates

Cells were washed twice in PBS and resuspended at 10^6 cells/ml in 1X sample buffer and immediately boiled for five minutes prior to loading on SDS PAGE gels.

Tissue homogenization

Tissues were quickly excised and homogenized in 5 volumes of 50 mM Tris HCl, 0.16 M NaCl, 1 mM EDTA, 1 mM PMSF, 1 μM pepstatin A, pH 8, using a Polytron homogenizer (Brinkman Instruments) at 4 °C. Protein

concentrations were measured according to Markwell *et al.* (1978). Tissue homogenates were stored as aliquots at -70 °C.

Erythrocyte membrane preparation

Rat erythrocyte membranes were prepared by a method adapted from Steck and Kant (1974; Cowell *et al.*, 1997). Briefly, packed erythrocytes were washed in PBS and centrifuged. Packed human, rat or bovine erythrocytes were rapidly diluted 1:20 in ice cold lysis buffer (5 mM Na_2HPO_4 pH 8, 0.5 mM PMSF, 1 μM pepstatin A) with stirring on ice. Membranes were pelleted by centrifugation for 10 minutes at 12 000 rpm (22 000 g) at 4°C in a JA14 rotor (Beckman). Supernatants, insoluble proteins and white blood cells sticking to the sides of the flasks were removed by tap aspiration. The pellets were repeatedly washed and centrifuged until the supernatant was colourless and the membranes were whitish in colour. The membranes were stored at -70 °C.

Triton X-114 extraction

Plasma membrane proteins were concentrated by Triton X-114 extraction (Bordier, 1981; Ko and Thompson, 1995). Briefly, cells were resuspended in 1% Triton X-114 on ice for 10 minutes. Samples were then heated to 37°C for 5 minutes to allow for phase separation. The detergent phase was pelleted by centrifugation at 15 000 rpm in a benchtop microfuge for 5 minutes. The resulting detergent phase, which was 12 % Triton X-114, was diluted back to 1% Triton X-114 by adding 20 mM HEPES pH 7.4, and phases were again separated as

described above. The proteins in the washed detergent phase were then precipitated by adding 5 volumes of -80°C acetone and incubating at -20°C for 20 minutes. The precipitated proteins were collected by microfugation, the acetone was aspirated and the pellet was dried at room temperature. The dried pellet was then resuspended in 1X sample buffer for SDS PAGE.

Electrophoresis

Polyacrylamide gel electrophoresis

Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) carried out by the method of Neville using a Mini Protean II gel apparatus (1971). Samples were prepared in sample buffer and boiled for 3 minutes prior to loading on the gels. A 12 % separating gel and a 3 % stacking gel were used. The gels were run at 16 mA until the dye front reached the stacking gel, and then at 40 mA until the dye front reached the bottom of the gel. Gels were either silver stained, or transferred to nitrocellulose as described below.

Silver staining of gels

Gels were fixed for 30 minutes in 50 % methanol, 10 % acetic acid with shaking. Fixed gels were rehydrated in 10% methanol, 10 % acetic acid with heating in a microwave for 1 minute, and this was followed by washing at room temperature for 5 minutes in dH_2O. Proteins in the gels were reduced in 33 μM dithiothreitol for 1 minute in the microwave. The gels were rinsed with water and

stained for 15 minutes in 0.1 % (w/v) $AgNO_3$. After staining the gels were rinsed thoroughly in dH_2O and then developed in 3 % (w/v) Na_2CO_3, 0.037 % (v/v) formaldehyde. Development was stopped by adding 5 % acetic acid after the bands were visible.

Western blotting

Proteins separated by SDS PAGE were blotted onto 0.2 μm nitrocellulose at 90 V for 50 minutes in a chilled BIORAD mini-cell transfer blotting apparatus.

N-terminal sequencing gels

All glassware was extensively washed in a solution containing 10 % HF and 90 % HNO_3, while plasticware including the gel and blotting apparatus was washed in Decon solution (prepared according to the manufacturer's instructions). Glassware and plasticware were thoroughly rinsed in distilled water before use. A 10 % SDS-polyacrylamide gel was prepared according to Laemmli (1970) and left to polymerize at 4°C overnight. A 3 % stacking gel was overlaid and allowed to polymerize for 2 hours. The sample containing the protein of interest was boiled in sample buffer and then loaded into the center well. This well was flanked by prestained standards and a smaller aliquot of the same sample in separate lanes. The gel was run at 20 mA until the dye front reached the bottom of the gel. The gel was then blotted onto PVDF at 90V for 50 minutes. The lane containing the protein of interest was then cut out and stained in 0.1 % Coomassie Blue in 50 % methanol and to visualize the 110-kDa protein. This band was then

cut out, rinsed three times in dH_2O and given to Sandy Kielland (University of Victoria) for N-terminal sequencing. The lanes containing the standards and the small amount of sample were cut away and developed by sandwich Western blotting to confirm the localization of the protein of interest.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in TAE buffer. Gels were 1.5 % agarose in TAE buffer containing 0.5 μg/ml ethidium bromide. They were run at 90 V until the dye front had almost reached the bottom of the gel, then transilluminated with UV light and the images captured using the ChemiImager 4000 gel documentation apparatus.

Detection of proaerolysin binding

Detection of proaerolysin-binding proteins by sandwich western blotting

Samples were separated by SDS-PAGE and blotted onto nitrocellulose as described above. Blots were blocked with 5 % skim milk in PBS containing 0.5 % Tween-20 (PBS-Tween). All subsequent incubations were in PBS-Tween. The blots were incubated for 1 hour with 20 nM proaerolysin, and then with polyclonal anti-aerolysin and anti-rabbit horseradish peroxidase (HRP) conjugate. In between each 1 hour incubation, blots were washed 3 times for 5 minutes in PBS-Tween. After the final 1 hour incubation, blots were washed for 1 hour in PBS-Tween with eight changes of buffer and then developed by enhanced chemiluminescence (ECL; Amersham Corp.).

The surface protein gp63, expressed in CHO cells or in *Leishmania major* samples was detected with a monoclonal anti-gp63 antibody followed by an anti-mouse horseradish peroxidase. Blots were developed by ECL.

Detection of proaerolysin bound to cells by flow cytometry

One milliliter of cells at 10^6 cells/ml with (EL4) and without GPI-anchored proteins (EL4 (Thy-1-f)) or EL4 cells that had been treated with and without methyl-β-cyclodextrin for 30 minutes at 37°C were exposed to 10^{-8} M FLAER in PBS for 30 minutes on ice. Cells were then washed twice with 1 ml of PBS and analyzed by flow cytometry.

Confocal microscopy

Murine lymphomas EL4 and EL4 (Thy-1-f) were suspended at 10^6 cells/ml in DMEM, 0.5 % BSA and labeled with 10^{-8} M wild-type or variant FLAER as indicated in Figure 28 for 1 hour at 4°C. When looking for changes in aerolysin distribution on the cell surface over time, prior to fixation cells were incubated at 37°C for 30 minutes. They were then washed twice in PBS and fixed with 4 % paraformaldehyde in PBS for 30 minutes at room temperature. The cells were then washed twice in PBS and visualized with a laser scanning confocal microscope after 1:1 dilution with 2.3 % DABCO, in 20 mM Tris, pH 8, 90 % glycerol.

Receptor Characterization

N-deglycosylation of glycoproteins

An equal volume of N-glycosidase incubation buffer (40 mM NaH_2PO_4, 100 mM EDTA, 1 % sodium dodecyl sulfate, 10 % β-mercaptoethanol, pH 7.5) was added to 10 µl of 4.5 x 10^6 EL4 T lymphomas/ml in PBS or 10 µl of supernatant from PI-PLC treated mouse brain homogenate, prepared as described previously. Samples were then boiled for 2 minutes. After cooling to room temperature, 3.3 µl of a protease inhibitor mixture consisting of 0.6 mM PMSF, 60 µg/ml aprotinin, 120 µM leupeptin and 12 µM pepstatin A was added, followed by 2.5 µl of 10 % octylglucopyranoside and 7.5 µl of peptide-N-glycosidase F (PNGaseF, Oxford Glycosystems) containing 1.5 units of the enzyme. A control incubation was also carried out in which 7.5 µl of buffer were added in place of the enzyme. After 18 hours at 37°C, sample buffer was added and aliquots were separated by SDS-PAGE and sandwich Western blotted.

Phosphatidylinositol-specific phospholipase C treatment

Five hundred microliters of EL4 T lymphomas at 10^8 cells/ml were incubated with 200 milliunits of PI-PLC for 2 hours at 37°C in PBS. A control sample was incubated without enzyme. Cells were diluted 1:1 in 0.1 % trypan blue in PBS and live/dead cell counts were performed using a hematocytometer to ensure there was no cell lysis. The cells were subsequently pelleted at 80 000 rpm (279 000 g) for 20 minutes at 4°C in a Beckman TLA100.2 rotor. Aliquots of the supernatants and cells were used for the sandwich Western blotting procedure.

Rat brain homogenate (1.0 ml) was treated with 400 mU PI-PLC at 37°C for 1 hour. Rat, human and bovine erythrocytes (4 mg protein/ml as determined by Markwell *et al.* (1978)) were treated with 300 mU/ml PI-PLC at 37°C for 30 minutes. In both cases the supernatants and cells or membranes were separated by centrifugation and analyzed by sandwich Western blotting.

Oligomerization studies

Trypsin treatment of proaerolysin

Proaerolysin (wildtype and variants) were activated according to standard lab procedures (Garland and Buckley, 1988). Briefly, a 10:1 (w:w) mixture of protoxin and trypsin was incubated for 15 minutes at room temperature.

Oligomer formation by aerolysin variants

Each trypsin-activated aerolysin variant was added to rat erythrocyte membranes to the final concentrations as indicated in the figure legends. After incubation at room temperature for 30 minutes, the membranes were collected by centrifugation at 15 000 rpm in a benchtop microfuge for 1 minute at 4°C, washed in HBS and recentrifuged. The final membrane pellets were resuspended in 1X sample buffer and aliquots were separated by SDS PAGE and blotted.

Cell Viability Assays

Cytotoxicity assays (EL4, AKR1)

Cells in complete Dulbecco's modified Eagle's medium were aliquoted at 1 x 10^6 cells/ml into 96-well microtiter plates and incubated with indicated proaerolysin concentrations for 1 hour at 37 °C and 5 % CO_2. Cell viability was assessed by the addition of MTS and PMS to a final concentration of 333 μg/ml MTS and 7.66 μg/ml PMS. The plates were incubated at 37 °C, 5 % CO_2, for 4 hours, after which A_{490} was measured.

In assays comparing the susceptibility of cells to aerolysin with and without PI-PLC treatment, 5 ml of 1 x 10^6 cells/ml were treated with 500 milliunits PI-PLC per ml for 2 hours at 37 °C rotating end over end. Control cells were incubated without enzyme. Cells were then pelleted by brief centrifugation, the supernatant was removed, and cells were resuspended in 5 ml DMEM containing 10 % fetal bovine serum. Cells (100 μl of 10^6 cells/ml) were then incubated with specified toxin concentrations for 1 hour at 37 °C as described above.

Erythrocyte lysis analysis

Spectrophotometric analysis of erythrocyte hemolysis

Aerolysin-induced erythrocyte hemolysis was assessed over a range of toxin concentrations. Activated aerolysin was added to 1 % rat erythrocytes to the

final concentrations indicated. Cells were incubated for 1 hour at 37°C with gentle rocking. Following incubation, unlysed cells and membranes were pelleted by microcentrifugation for 30 seconds. The extent of cell lysis was estimated by comparing the absorbance of released hemoglobin in the supernatant at 560 nm with the absorbance of supernatant from cells lysed completely with 0.1 % sodium dodecyl sulphate.

Kinetics of toxin-induced hemolysis

Activated aerolysin at a final concentration of 5 nM was added to stirred plastic cuvettes containing washed 0.8 % v/v human erythrocytes in 1.5 ml PBS. The rate of hemolysis was monitored by measuring the decrease in optical density of the erythrocyte suspensions at 600 nM and 37°C as a function of time. Readings were made using a Varian Cary I recording spectrophotometer.

GPI-anchored protein purification

Contactin Purification

Rat brain was homogenized (20 % w/v) in 50 mM Tris, 160 mM NaCl, 1 mM EDTA, pH 8, containing 1 μg/ml pepstatin A and 1 mM PMSF at 4°C. This homogenate (20 ml) was centrifuged at 14 000 x g for 10 minutes. The resulting pellet was resuspended in the same buffer and recentrifuged. The washed pellet

was resuspended in the original volume of starting buffer and 4 units of PI-PLC were added. Following incubation at 37°C for 2 hours rotating end over end, the sample was recentrifuged and the resulting supernatant was applied to a lentil lectin column. Fractions containing contactin were eluted from the column with 1 M α-methyl-D-mannoside and applied to a 40 ml column of DEAE-sepharose CL-6B equilibrated in 10 mM Tris pH 8. A 400 ml gradient was run from 10 mM Tris pH 8 to10 mM Tris pH 8, 0.5 M NaCl. Contactin was eluted after 80 ml of the gradient had been applied to the column.

PLAP Purification

Twenty five milligrams of crude human placental alkaline phosphatase (PLAP) powder were dissolved in 50 ml of 1% Triton X-114 in PBS containing 1 mM phenylmethylsulfonylfluoride. by incubating for 20 minutes on ice. The extract was separated into detergent-rich and aqueous phases by warming the sample to 37°C for 10 minutes and then centrifuging in a JA-17 rotor (Beckman) for 10 minutes at 10 000 rpm (14 000g) and 23°C. The detergent-rich phase was cooled and diluted back to 1 % Triton X-114 by adding cold 20 mM HEPES, pH 7.4. Following warming and centrifuging to separate the phases once more, protein was precipitated from the detergent-rich phase by adding 5 volumes of -80°C acetone and incubating on ice for 30 minutes. Precipitated protein was collected by centrifugation at 5000 rpm (3500 g) for 30 minutes at 0°C in a JA17 rotor. The acetone was decanted and the pellet was dried for 2 hours under vacuum. The dried pellet was resuspended in 20 mM HEPES, pH 7.4, containing 1 % octylglucoside, and applied to a DEAE column equilibrated in the same

buffer. The column was eluted with a salt gradient of 0 to 0.5 M NaCl in 20 mM HEPES, pH 7.4, 1 % octylglucoside. Enzyme activity, which was assayed using a standard alkaline phosphatase assay (Sigma), appeared at approx. 0.18 M salt. Silver staining after SDS-PAGE electrophoresis produced a single band accounting for more than 95 % of applied material. The purified protein had a specific activity of approximately 400 U/mg, slightly less than the specific activity reported previously for purified alkaline phosphatase lacking the GPI anchor (Chang *et al.*, 1992).

Using liposomes to study aerolysin induced lysis

Liposome preparation

Lipid films containing 12 μmol of total lipid in each of the following molar proportions, 5PC:3PE:3CH, 3PC:3PE:3CH:2SM, 4PC:3PE:3CH, and 3PC:3PE:3CH:1SM, were dried under nitrogen. Dried films were desiccated overnight, and then each rehydrated in 2 ml of 20 mM HEPES, 0.15 M NaCl, 100 mM carboxyfluorescein, pH 7.4. Liposomes containing sphingomyelin were rehydrated at 45°C, while the others were rehydrated at room temperature. Liposomes were rapidly frozen (-70°C acetone bath) and thawed (45°C water bath) six times. After freeze thawing, liposomes were passed through a 0.4 μm polycarbonate filter (Nucleopore) six times, using a Lipex Biomembrane extruder. The sphingomyelin liposomes were extruded at 45°C and the others at room temperature. Lipid phosphorous was measured according to established methods (Bartlett, 1959).

Reconstitution of PLAP into liposomes

To determine optimum octylglucoside concentrations for incorporation of GPI-anchored proteins into liposomes, the method of Nosjean and Roux, (1999) was used. For these studies liposomes were produced without carboxyfluorescein as it interfered with absorbance at 450 nm. The turbidity of liposomes at 450 nm was monitored while increasing the concentration of octylglucoside until the absorbance at 450 nm began to drop. This detergent concentration, 20 mM for 4PC:3PE:3CH, 21 mM for 3PC:3PE:3CH:1SM, 23 mM for 5PC:3PE:3CH, and 30 mM for 3PC:3PE:3CH:2SM liposomes, was chosen for PLAP incorporation. Approximately 10 μg of PLAP was incubated with 500 μl of 1.3 mM lipid. The mixtures were dialyzed overnight against 20 mM HEPES, 0.15 M NaCl pH 7.4 at 4°C (5PC:3PE:3CH liposomes), or 22°C (3PC:3PE:3CH:2SM liposomes) to remove detergent and free carboxyfluorescein. Liposomes were then passed over a Sephacryl S-300 column (23 ml) equilibrated in 20 mM HEPES, 0.15 M NaCl, to remove unincorporated PLAP and free dye. Phosphorous assays were performed on liposomes collected off the column. Liposomes were sized using a Nicomp submicron particle sizer.

Liposome release assay

Carboxyfluorescein release was measured at 22 or 37°C as indicated using a Photon Technology QM-1 spectrofluorimeter. The excitation wavelength was set at 490 nm and the emission wavelength at 520 nm. A 5 nm slit width was used

for both monochromators. Aliquots of liposome preparations (0.26 nmol lipid) were added to 3 ml of 20 mM HEPES, 0.15 M NaCl, pH 7.4. Activated aerolysin was added to the indicated concentrations at 1 minute and carboxyfluorescein-release was followed with time.

Studying the involvement of raft domains in aerolysin activity

Cholesterol extraction with methyl-β-cyclodextrin

EL4 cells at 2 x 10^6 cells/ml were washed twice in neat DMEM and then incubated with or without 10 mM methyl-β-cyclodextrin in DMEM for 30 minutes at 37°C, rotating end over end. Following extraction, half of the cells were washed twice in DMEM and used in the cytotoxicity assay and the FACS binding assay; the other half were washed twice in PBS and then a cholesterol determination (Cholesterol 20, Sigma Diagnostics) was performed according to the manufacturer's directions. Briefly, 2 ml of chloroform: methanol (1:2) was added to 5 x 10^6 cells/ml in 0.5 ml of PBS and mixed. To this, 0.65 ml of chloroform followed by 0.65 ml of dH_2O was added and mixed. This mixture was centrifuged to separate the phases. The upper phase was removed and the lower phase was transferred to a clean tube and dried under nitrogen. The film was then rehydrated in 80 µl of dimethyl formamide (DMF), and 20 µl of this material was mixed with 1 ml of the cholesterol assay reagent. The samples were incubated at 37°C after which the absorbance at 500 nm was read.

Washed human erythrocytes were treated with 3.5 mM methyl-β-cyclodextrin or PBS alone and then washed with PBS. The amount of cholesterol

extracted was determined with a Cholesterol 20 assay as described above. Aerolysin induced hemolysis was monitored spectrophotometrically over time as described above.

Analysis of detergent insoluble material

Lymphomas (1 ml of 2 x 10^7 cells/ml in DMEM, 0.5 % BSA) were incubated with 10^{-8} M Y221G proaerolysin on ice for one hour. This proaerolysin variant was used here because it can bind as well as native proaerolysin, but does not cause cell death as it is deficient in insertion or the formation of a functional channel (unpublished data). Following incubation, the cells were washed twice in PBS and then extracted with 500 µl of 25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100 (w/v), containing a protease inhibitor cocktail (1 mM phenylmethylsulfonylfluoride, 2 µg/ml aprotinin, 2.2 µg/ml leupeptin and 1 µg/ml pepstatin A) for 30 minutes on ice (Fra *et al.*, 1994). In some experiments the detergent extraction step was carried out first. Then 10^{-8} M Y221G was added to the cells and the mixtures were incubated for 15 minutes at 4°C. Mixtures were adjusted to 1.5 M sucrose (w/v) and overlaid with 8 ml of 1.2 M sucrose (w/v), 10 mM Tris, pH 7.4, followed by 2.5 ml of 0.15 M sucrose, 10 mM Tris pH 7.4. The samples were then centrifuged in an SW41 rotor at 38 000 rpm (221 000 g) for 18 hours at 4°C. Fractions of 1 ml were collected and sample buffer was added for SDS-PAGE. Material at the bottoms of the centrifuge tubes was suspended directly in sample buffer.

The detergent solubility of T lymphoma Thy-1 was determined by simply measuring pelleting of Thy-1 after detergent extraction (Benting *et al.*, 1999). Cells were extracted with 1 % (w/v) Triton X-100 in PBS with protease inhibitors for 15 minutes on ice as above. This was followed by centrifugation in a TA100.2 rotor (Beckman) at 75 000 rpm (245 000 g) at 4°C for 30 minutes. Protein was precipitated from the supernatant and the resuspended pellet with trichloroacetic acid (10 % TCA wt/vol). The dried pellet was resuspended in 1X sample buffer and neutralized with saturated Tris. Thy-1 was detected by sandwich Western blotting.

The detergent insolubility of PLAP incorporated into liposomes was also measured by centrifugation. Liposomes (100 nmol of lipid) were pelleted at 75 000 rpm (245 000 g) at 4°C for 20 minutes in a TA100.2 rotor and the pellet was resuspended in 450 µl of 0.1 % Triton X-100 in 20 mM HEPES, 0.15 M NaCl and extracted on ice for 15 minutes. A control sample was incubated in buffer without detergent. After centrifugation at 75 000 rpm (245 000 g) at 4°C for 30 minutes, the supernatants were removed and the pellets were resuspended in the same buffer. Both supernatants and resuspended pellets were then precipitated with trichloroacetic acid and Thy-1 was detected by sandwich Western blotting.

Apoptosis Assays

DNA fragmentation and caspase-3 activity

EL4 T lymphomas at 10^6 cells/ml were incubated with the indicated proaerolysin concentrations overnight (18.5 hrs) and then analyzed for DNA

fragmentation and caspase-3 protease activity and cell viability with MTS and PMS as described above.

DNA fragmentation was analyzed by the method of Martin *et al.*, (1990). Briefly, 10 ml of cells at 10^6 cells/ml treated with proaerolysin were microfuged for 5 minutes at room temperature. The supernatant was discarded and cells were washed twice in PBS. Pellets were then resuspended at 2 x 10^7 cells/ml in lysis buffer containing 50 mM Tris, pH 8, 10 mM EDTA, 0.5 % lauroylsarcosine, 0.5 mg/ml proteinase K and incubated for 1 hour at 50°C. The entire cell lysate was then extracted with 1 volume of phenol-buffered Tris, pH 7.4. Samples were inverted to mix and then microfuged to separate phases. The supernatants were reextracted with phenol and then washed twice with choloform:isoamyl alcohol (24:1 v/v) as above. The supernatants were removed and 2.5 volumes of 10 mM Tris pH 8, 1 mM EDTA (TE) was added, followed by 2 volumes of -70°C 95 % ethanol and placed at -70°C for 1 hour. Samples were then microcentifuged for 10 minutes at room temperature. The supernatants were then removed and the pellets were air dried for 15 minutes. Dried pellets were resuspended in TE buffer and diluted in sample buffer for agarose electrophoresis.

Caspase-3 protease activity was measured with a CPP32 protease assay according to the manufacturer's directions. Briefly, 2 x 10^6 cells treated with proaerolysin were pelleted by microcentrifugation. Cells were resuspended in 50 µl of chilled cell lysis buffer supplied by Clontech. To this, 50 µl of 2X reaction buffer and 5 µl of 1 mM conjugated substrate were added. Samples were

incubated for 1 hour at 37°C. Finally, samples were diluted to 1 ml in dH_20 and the absorbances were read at 400 nm.

Flow cytometric measurement of changes in intracellular calcium

EL4 cells were washed twice in imidazole buffered saline (145 mM NaCl, 10 mM glucose, 5 mM KCl, 5 mM imidazole, pH 7.4), resuspended in the same buffer containing 2 μM Fluo3-AM (Molecular Probes) at 10^7 cells/ml, and incubated for 1 hour in the dark at room temperature. The cells were then washed twice in 20 mM HEPES, 150 mM NaCl, 10 mM glucose, 1 mM $CaCl_2$, 1 g/l BSA, pH 7.4 at 4°C and kept on ice. In experiments looking for changes in intracellular calcium as a result of liberation of intracellular calcium stores, cells were suspended in buffer lacking calcium. Just before use the cells were diluted to 10^6 cells/ml and treated with or without 10^{-9}, 10^{-10} or 10^{-11} M proaerolysin at room temperature. Samples were analyzed over time by flow cytometry using a Beckton Dickinson FACSCalibur with Cellquest software.

RESULTS

Characterization of proaerolysin receptors on nucleated cells

Proaerolysin binds to a 30-kDa protein on T lymphomas

A 47-kDa GPI-AP on rat erythrocytes was previously shown to be a receptor for aerolysin (Cowell *et al.* 1997). With the aim of identifying receptors for the toxin on nucleated cells, Western blots of mouse EL4 T lymphoma lysates were probed with low concentrations of proaerolysin (2×10^{-8} M), followed by a polyclonal anti-aerolysin antibody and an enzyme-linked secondary antibody. Using this sandwich technique, a 30-kDa protein that was heavily stained was identified (Figure 5, lane 1).

The 30-kDa proaerolysin-binding protein on T lymphomas is GPI-anchored

Since the rat erythrocyte aerolysin receptor (EAR) shares N-terminal sequence homology with an ADP-RT (Cowell *et al.* 1997), it was initially thought that the T lymphoma proaerolysin-binding protein might be Rt-6, because it is a 30-kDa GPI-anchored ADP-RT that is found on most T lymphomas (Bortell *et al.*, 1999). To determine whether or not the 30-kDa proaerolysin-binding protein on EL4 cells was GPI-anchored, a corresponding mutant cell line (EL4 (Thy-1-f)), which does not express GPI-APs due to a defect in an enzyme required for anchor biosynthesis, was used for comparison (Fatemi and Tartakoff, 1988). Sandwich Western blots of aliquots of these cells revealed that they did not

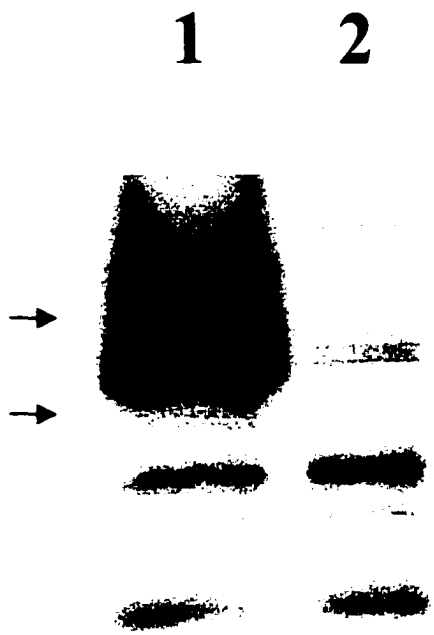


Figure 5. **Proaerolysin binding to proteins from EL4 cells and a corresponding cell line defective in GPI anchoring.** Lane 1, EL4 cells; lane 2, corresponding GPI-defective mutant EL4 (Thy-1-f). Equal numbers of cells (9000 cells/lane) were separated by SDS PAGE and blotted as described in the methods. Arrows indicate the positions of molecular mass markers. They are from top 33, and 29-kDa.

contain the 30-kDa protein found in the normal cell line (Figure 5, lane 2), indicating that this protein is GPI-anchored.

To confirm GPI-anchoring of the 30-kDa protein, EL4 cells were treated with PI-PLC, which selectively hydrolyses the phosphodiester bond on the anchor, releasing GPI-APs from the surfaces of most cells. Following treatment, there was a pronounced decrease in the amount of the 30-kDa protein associated with the cells (Figure 6). Instead it appeared in the supernatant after cell pelleting, indicating that it had been released from the cell surface (Figure 6).

Thy-1 is the 30-kDa proaerolysin-binding protein on T lymphomas

Several GPI-APs have been characterized on T lymphocytes (Boccuni *et al.*, 2000; Rosti, 2000), including two with molecular masses of approximately 30-kDa that were candidates for the band observed in Figure 6. One of these is Rt-6, mentioned above. However, this protein is not expressed by the strain of T lymphomas (EL4) used in these studies (Prochazka *et al.*, 1991). The other candidate was Thy-1, the most abundant GPI-AP found on the surface of T lymphocytes (Williams and Gagon, 1982). Thy-1 is N-glycosylated and its molecular mass when deglycosylated is 14.5-kDa (Parekh *et al.*, 1987). When EL4 T lymphomas were treated with peptide-N-glycosidase F, an enzyme that releases N-linked sugars from proteins, the molecular mass of the 30-kDa proaerolysin-binding protein observed in these cells was reduced to approximately 14.5-kDa (Figure 7), consistent with the conclusion that the protein is Thy-1. This result also demonstrated that N-linked sugars are not required for

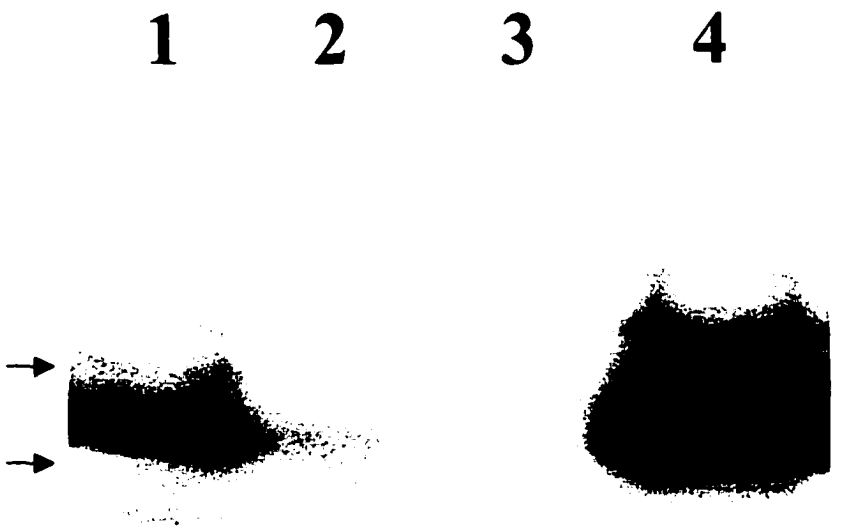


Figure 6. **The proaerolysin binding component of EL4 T lymphomas is a GPI-anchored protein.** EL4 cells were treated with or without the enzyme as described in the text. Lane 1, control incubation (no enzyme) of EL4 cells; lane 2, PI-PLC treated EL4 cells; lane 3, control incubation, supernatant; lane 4, PI-PLC treated cells, supernatant. Twice as much supernatant as cells (1.2×10^4) were separated by SDS-PAGE and sandwich Western blotted. Arrows indicate the positions of 33 and 29-kDa molecular mass markers.

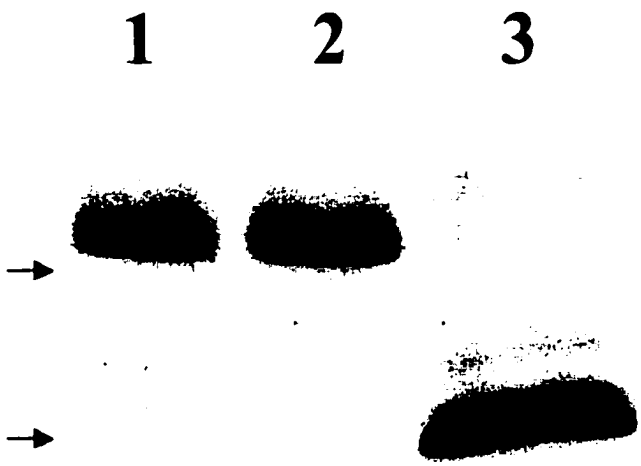


Figure 7. **N-linked sugars are not required for proaerolysin binding to the proaerolysin binding protein in T lymphomas**. See the text for details. Lane 1, untreated EL4 cells; lane 2, incubated control EL4 cells (no enzyme); lane 3, PNGase-F treated EL4. Arrows indicate the positions of 29 and 14-kDa molecular mass markers.

proaerolysin binding to this protein. A similar conclusion was reached previously with EAR (Gruber *et al.*, 1994; Figure 20).

Several mouse tissues were next screened for proaerolysin-binding proteins. The sandwich Western blot in Figure 8 shows that proaerolysin bound to proteins migrating at 33 and 44-kDa in kidney, and to 44-kDa proteins in heart and liver. These proteins have not been identified. In addition, an intensely staining band migrating at 110-kDa was observed in brain, the characterization of which will be described later. By far the most intense band that was detected in this experiment migrated at approximately 30-kDa in brain and thymus, corresponding to the band detected in T lymphomas. A less intense band migrating at the same molecular mass was also observed in spleen. Since Thy-1 is found in large amounts in brain and thymus (Williams and Gagnon, 1982), and to a lesser extent in spleen, this was more evidence that the 30-kDa protein detected in these tissues and in EL4 cells is Thy-1.

Unequivocal evidence that proaerolysin binds to Thy-1 came from a comparison of blots of a T lymphoma cell line that has the gene for Thy-1 knocked out (AKR1 (Thy-1-d)) with the corresponding parental cell line expressing Thy-1 (AKR1; Evans *et al.*, 1987). The results in Figure 9 demonstrate that proaerolysin bound to the same 30-kDa protein on the parental AKR1 T lymphoma cell line (Figure 9, lane 3) as on EL4 cells (Figure 9, lane 1). As expected, this protein was absent from the cell line not expressing Thy-1 (AKR1 (Thy-1-d); Figure 9, lane 4).

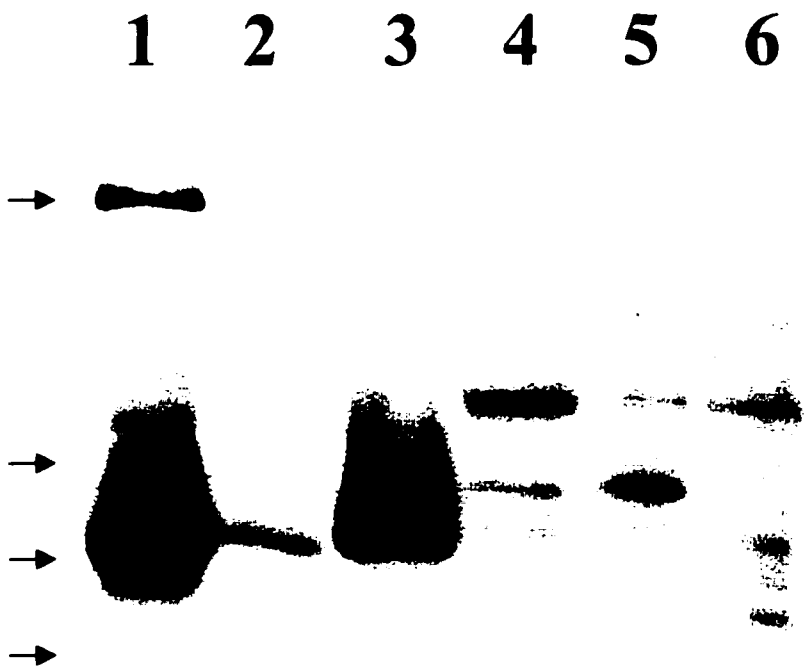


Figure 8. **Proaerolysin binding to proteins of mouse tissues.** Homogenates of mouse tissues were separated by SDS-PAGE and blotted as described in the materials and methods. Approximately the same amount of each tissue (300 μg tissue/lane) was applied. Lane 1, mouse brain; lane 2, spleen; lane 3, thymus; lane 4, heart; lane 5, kidney; lane 6, liver. Arrows indicate the positions of molecular mass markers. They are from the top 109, 49, 33 and 29-kDa.

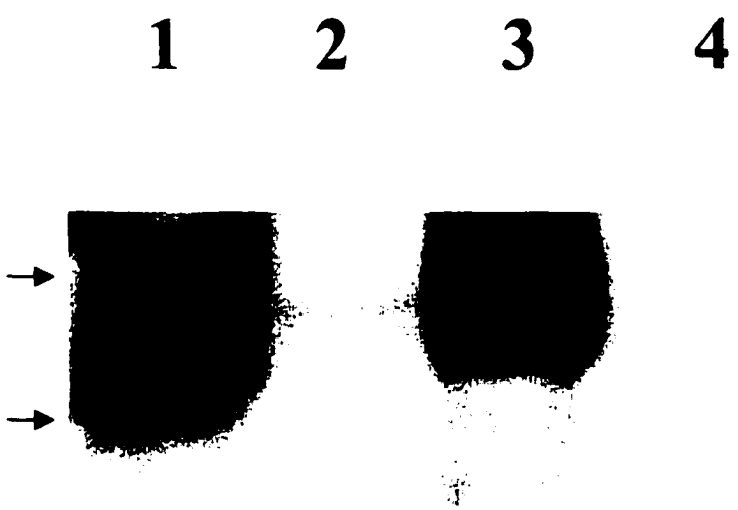


Figure 9. **Proaerolysin binding to proteins from parental T lymphoma cell lines and cell lines deficient in GPI-anchoring or unable to express Thy-1.** Lane 1, EL4; lane2, EL4 (Thy-1-f); lane 3, AKR1; lane 4, AKR1 (Thy-1-d). Approximately equal numbers of cells (9000 cells/lane) were applied. Arrows mark the positions of 33 and 29-kDa markers.

Sandwich Western blotting with proaerolysin is a sensitive method for detecting GPI-APs

I next wanted to determine how sensitive a method proaerolysin sandwich blotting was for detecting Thy-1 and EAR. As a comparison I chose a commercial antibody (anti-CRD antibody; Zamze *et al.*, 1988) that recognizes the GPI-anchors on proteins once the diacylglycerol is removed with PI-PLC. Although the level of sensitivity of this antibody is quite low, typically requiring 0.2 μg of GPI-AP, it was the only antibody available for comparison. To determine the sensitivity of proaerolysin detection, a blot containing varying amounts of purified rat brain Thy-1 was probed with proaerolysin (Figure 10). These results showed that picogram amounts of purified Thy-1 could be detected in this way, an indication that proaerolysin has a very high affinity for this protein. The strength of this interaction has recently been confirmed by surface plasmon resonance, which established that the binding constant for the interaction of proaerolysin with immobilized Thy-1 is approximately 2.0×10^{-8} M (MacKenzie *et al.*, 1999). Next, blots containing 0.2 μg of EAR and 1 μg of Thy-1, treated with and without PI-PLC, were used to compare proaerolysin sandwich blotting with the anti-CRD antibody. The results in Figure 11 show that the anti-CRD antibody was able to detect 0.2 μg of PI-PLC treated EAR and 1 μg of PI-PLC treated Thy-1 after a five minute exposure (Figure 11, lanes 2 and 4 panel A), whereas only a few seconds were needed to develop a blot containing the same samples with proaerolysin (Figure 11, panel B). In fact, the corresponding blot of Thy-1 samples could not be presented because the signal was so strong that it blackened

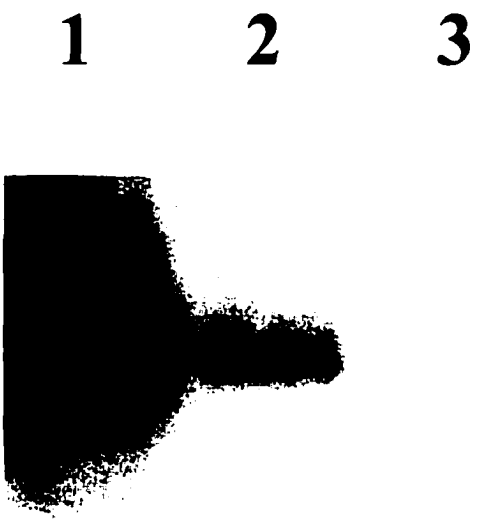


Figure 10. **Detection of purified Thy-1 using sandwich Western blotting with proaerolysin.** Lane 1, 30 ng; lane 2, 3 ng; lane 3, or 300 pg of Thy-1 were separated by SDS-PAGE and blotted to determine the minimum quantity of Thy-1 that could be detected by proaerolysin.

Figure 11. **Comparison of the ability of the anti-CRD antibody and proaerolysin sandwich Western blotting to detect GPI-anchored proteins.** Purified rat EAR (340 ng) or 1 μg of Thy-1 from rat brain were incubated with (lanes 2 and 4) and without (lanes 1 and 3) 150 mU of PI-PLC for 2 hours at 37 °C. After incubation, sample buffer was added and 200 ng of protein was run by SDS PAGE and blotted. A. The anti-CRD antibody (Oxford Glycosystems) was used to detect the GPI-anchors of rat EAR (lane 2) and Thy-1 (lane 4). B. The sandwich Western blotting procedure was used to detect untreated and PI-PLC treated EAR. A corresponding blot of Thy-1 is not shown as detection of 1 μg of Thy-1 by sandwich Western blotting generated too strong a signal.

the film after a flash exposure. The results in Figure 11 also show that only proaerolysin could detect protein that had not been treated with PI-PLC. These results demonstrate that proaerolysin is a much more sensitive and versatile method of detecting these proteins than the anti-CRD antibody.

Glycosylphosphatidyl-inositol anchored proteins are receptors for aerolysin

Having shown that aerolysin was able to bind to Thy-1 with high affinity, we wanted to determine if the interaction promoted channel formation. Gruber *et al.* had used planar lipid bilayers to demonstrate that the incorporation of EAR resulted in an increase in channel formation by aerolysin (1994). A comparable set of experiments was carried out by Dr. Skrikumar Raja, using liposomes containing entrapped dye (Nelson *et al.*, 1997). These results clearly showed that the presence of Thy-1 stimulated channel formation by aerolysin. Thus like EAR, Thy-1 is a functional receptor for aerolysin.

In light of the above result, it was surprising to discover that cells lacking Thy-1 (AKR1 (Thy-1-d)) were virtually as sensitive to aerolysin as cells that display the protein (AKR1; Figure 12). This suggested either that Thy-1 is not a receptor *in situ*, or that other surface molecules could also act as receptors. To determine if this was the case, cells with and without Thy-1 were treated with PI-PLC. Control cells were incubated without PI-PLC. The cells were then treated with proaerolysin. The results in Figure 12 show that cells lacking Thy-1 are 10^2-fold less sensitive to aerolysin following PI-PLC treatment, indicating that one or more GPI-AP that can function as an aerolysin receptor had been removed.

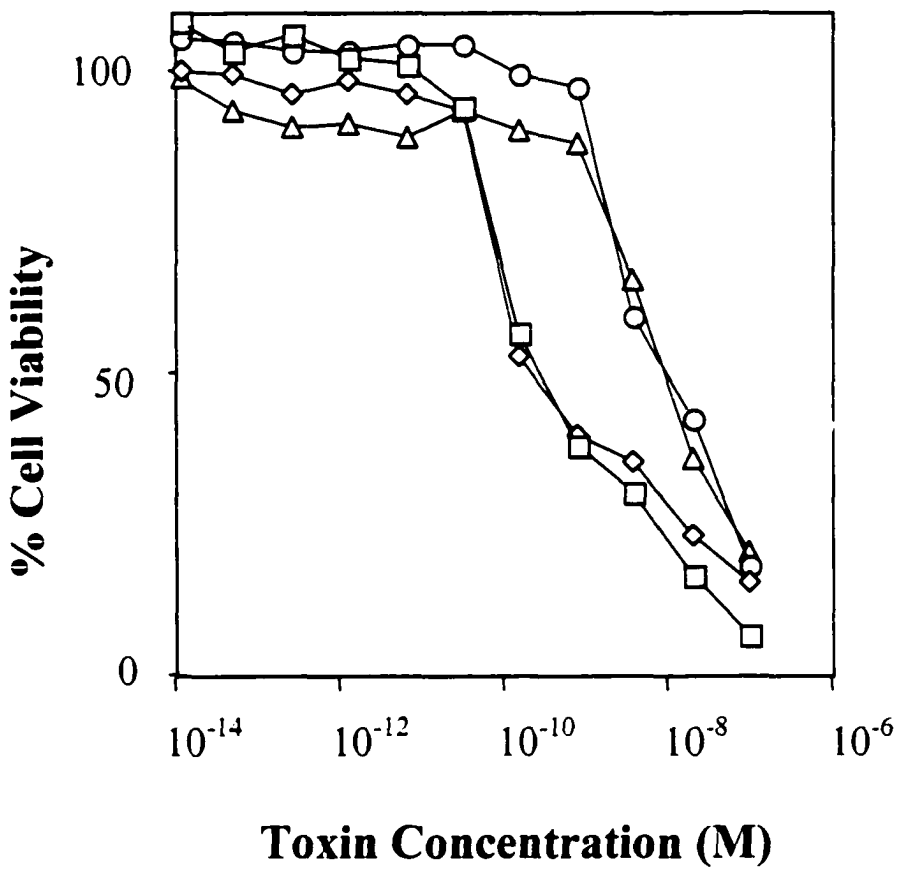


Figure 12. **Mouse T lymphomas contain more than one GPI-anchored aerolysin receptor.** Sensitivity of AKR1 cells to aerolysin before (□) and after PI-PLC treatment (o) as compared to the sensitivity of AKR1 (Thy-1-d) cells before (◇) and after treatment with PI-PLC (△) as described in the methods.

Although it was now clear that T lymphomas contain more than one GPI-anchored receptor, sandwich Western blots of cell lysates only detected Thy-1 (Figure 9). This may be because some GPI-APs cannot be detected by sandwich Western blotting or because the amount of an individual GPI-AP in a cell lysate loaded in the lane is too small to detect. Proaerolysin can detect as little as 300 pg of Thy-1 by sandwich Western blotting (Figure 10). Hence, assuming comparable binding affinities, in order to detect a GPI-AP in a cell lysate of 10^{4} cells the protein would have to be present at approximately 5×10^{5} copies/cell. Since the copy number for a given GPI-AP on T lymphocytes can vary from 2×10^{4} copies/cell for CD59 to up to 10^{6} copies/cell for Thy-1 (Brooimans *et al.*, 1992; Fletcher *et al.*, 1992; Williams and Gagon, 1982), it is not surprising that proaerolysin fails to detect every GPI-AP in cell lysates. To concentrate proteins for detection by sandwich Western blotting, AKR1 and AKR1 (Thy-1-d) cells were first extracted with Triton X-114. This detergent solubilizes GPI-anchored and other hydrophobic proteins (Ko and Thompson, 1995). The hydrophobic proteins are then separated from water soluble proteins by warming the lysate to a temperature above the cloud point of the detergent. At this temperature detergent micelles aggregate and come out of solution, forming a separate phase that contains most of the detergent as well as the GPI-APs (Terstappen *et al.*, 1993). The proteins in the detergent phase from solubilized AKR1 cells were further concentrated by TCA precipitation. They were then dissolved in sample buffer, separated by SDS PAGE and sandwich Western blotted. The results in Figure 13 show that previously unseen proaerolysin-binding proteins were revealed in the

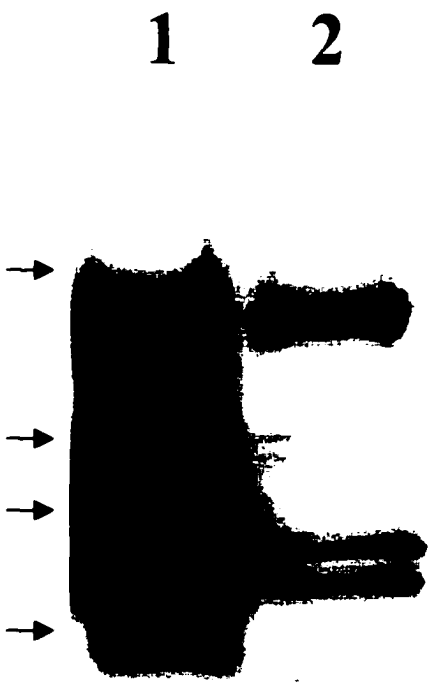


Figure 13. **Analysis of acetone precipitated Triton X-114 extracts for GPI-anchored proaerolysin binding proteins.** AKR1, lane 1, and AKR1 Thy-1-d; lane 2, were extracted with Triton X-114 and the detergent phase was acetone precipitated and separated by SDS PAGE for sandwich Western blotting as described in the methods. Arrows mark the positions of 80, 51, 34 and 27-kDa markers.

AKR1 (Thy-1-d) cell line after concentration. These proteins are masked by the dark band corresponding to concentrated Thy-1 in the parental cells. Further work will be required to determine if they are indeed GPI-APs and if they facilitate aerolysin activity.

The contribution of GPI-APs to aerolysin activity on T lymphomas

Phosphatidylinositol specific phospholipase C treatment of cells never results in 100 % release of GPI-APs (Low, 1987; Wong and Low, 1994). To obtain a more accurate sense of the role that these proteins were playing in aerolysin activity than was obtained from the results in Figure 12, the sensitivity of EL4 (Thy-1-f) cells was compared to that of normal cells (EL4). The results in Figure 14 demonstrate that the cell line lacking GPI-APs was 10^{4}-fold less sensitive to aerolysin than the corresponding parental cell line. This suggests that GPI-APs were in some way facilitating aerolysin activity.

The large difference in the sensitivity of cells plus and minus GPI-APs to aerolysin should be reflected by a difference in the amount of aerolysin bound to these cells. To determine if this was the case, EL4 and EL4 (Thy-1-f) cells were labeled with 10^{-8} M FLAER T253C/A300C and analyzed by confocal microscopy and flow cytometry. As expected, both techniques revealed that normal cells bound much more proaerolysin than those lacking GPI-APs (Figures 15 and 16).

In addition to binding aerolysin to cell surfaces it was possible that GPI-APs were promoting channel formation in some other way. It might be expected that aerolysin bound to GPI-APs would have a different lateral mobility than toxin

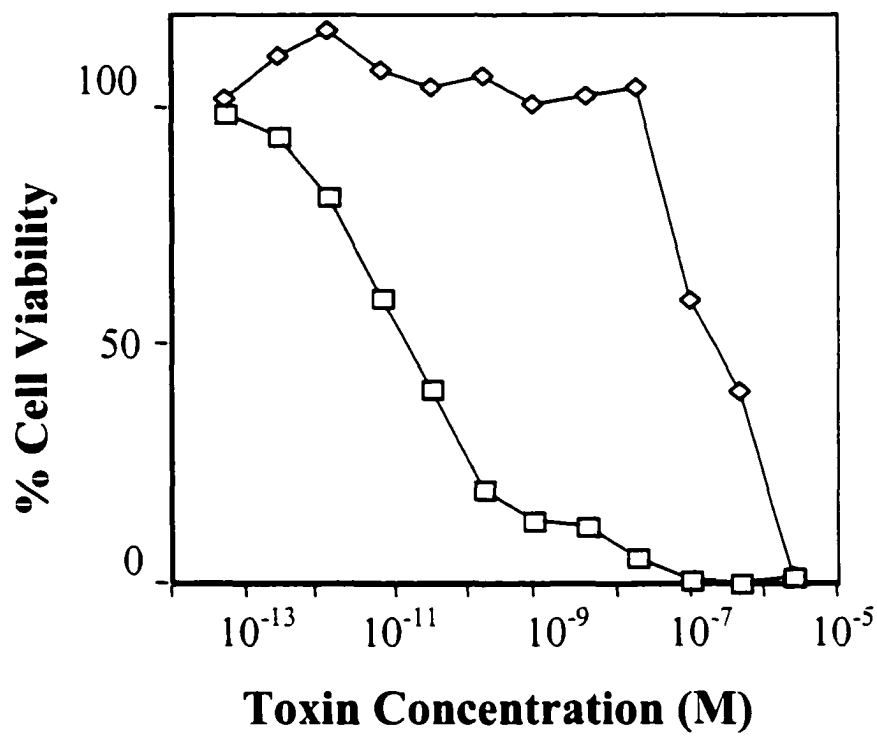


Figure 14. **EL4 cells lacking GPI-anchored proteins are less sensitive to aerolysin**. Parental EL4 cells (□) and the corresponding mutant cell line EL4 (Thy-1-f; ◇) not expressing GPI-anchored proteins at 10^6 cells/ml were treated with a range of proaerolysin concentrations as indicated. Cell viability was determined using an MTS/PMS viability assay as described in the methods.

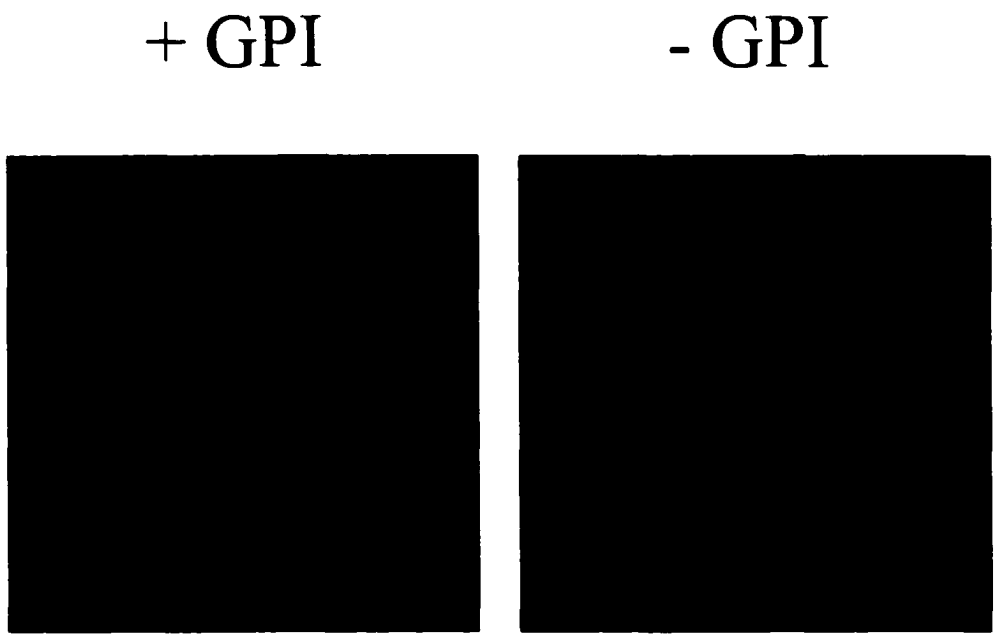


Figure 15. **Confocal microscopy analysis of aerolysin binding to cells with and without GPI-anchored proteins.** EL4 (+ GPI) and EL4 (Thy-1-f; - GPI) were labeled with 10^{-8} M T253C/A300C FLAER proaerolysin and visualized by confocal microscopy as described in the methods.

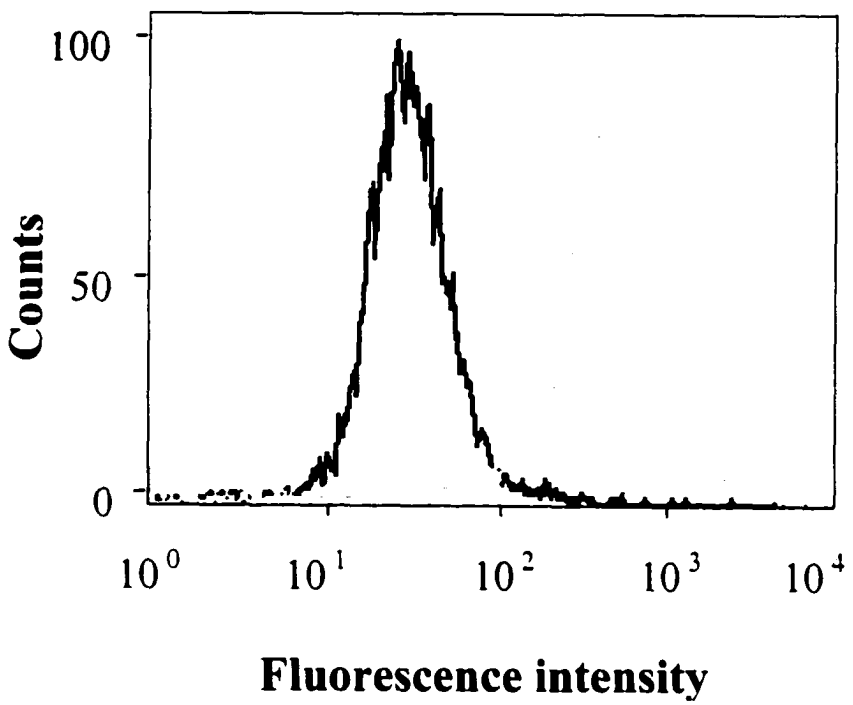


Figure 16. **More FLAER binds to cells with GPI-anchored proteins than to those without.** EL4 (gray line), and EL4 (Thy-1-f; black line) cells were labeled with 10^{-8} M Y221G FLAER as described in the methods section and were analyzed by flow cytometry.

bound to transmembrane proteins due to the fact that some GPI-APs, including Thy-1, have a faster diffusion coefficient than transmembrane proteins (Gall and Edelman, 1981; Ishihara et al., 1987; Kooyman *et al.*, 1995; Noda *et al.*, 1987; Vaz *et al.*, 1984; Zhang *et al.*, 1991 and 1992). This increased lateral mobility might promote oligomerization by increasing the chance that aerolysin molecules would encounter one another on the cell surface. To determine if this was a possibility, toxin-labeled cells plus and minus GPI-APs were incubated for 1 hour at 37°C to allow for oligomerization. These cells had been incubated with a high concentration of proaerolysin to maximize binding allowing for comparable binding to cells with and without GPI-APs. Samples of cells were then washed, dissolved in sample buffer, separated by SDS PAGE and blotted to detect oligomer. The results in Figure 17 demonstrate that there was indeed more oligomer formed on cells with GPI-APs even though binding to both cell types was comparable under the conditions used. Enhanced oligomerization following binding is likely another factor contributing to the difference in aerolysin sensitivity between cells containing or lacking GPI-APs.

Identification of another proaerolysin-binding GPI-AP

The nature of the 110-kDa protein previously detected in brain tissue by sandwich blotting was next investigated. Treatment of rat brain homogenate with PI-PLC resulted in a decrease in the amount of this protein associated with cells. It instead appeared in the supernatant along with Thy-1 following cell pelleting (Figure 18, lane 6), indicating that it too is GPI-anchored. Two GPI-APs that

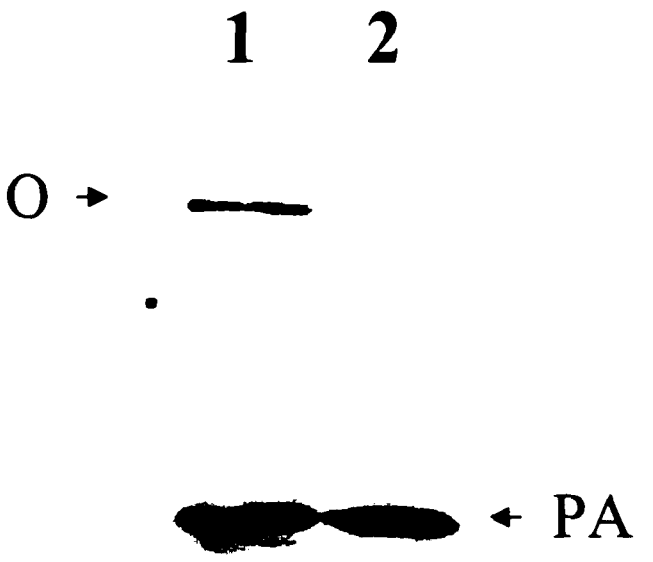


Figure 17. **Oligomerization of aerolysin on the surface of cells containing or lacking GPI-anchored proteins.** EL4 cells or EL4 (Thy-1-f) cells at 10^6 cells/ml were incubated for 1 hour at 37 °C with 5 x 10^{-7} M Y221G proaerolysin, 0.5 % BSA in PBS. Cells were washed once in PBS and resuspended in sample buffer. Aliquots of cells (10^4 cells/lane) were separated by SDS PAGE and Western blotted to detect proaerolysin (PA) and oligomer (O).

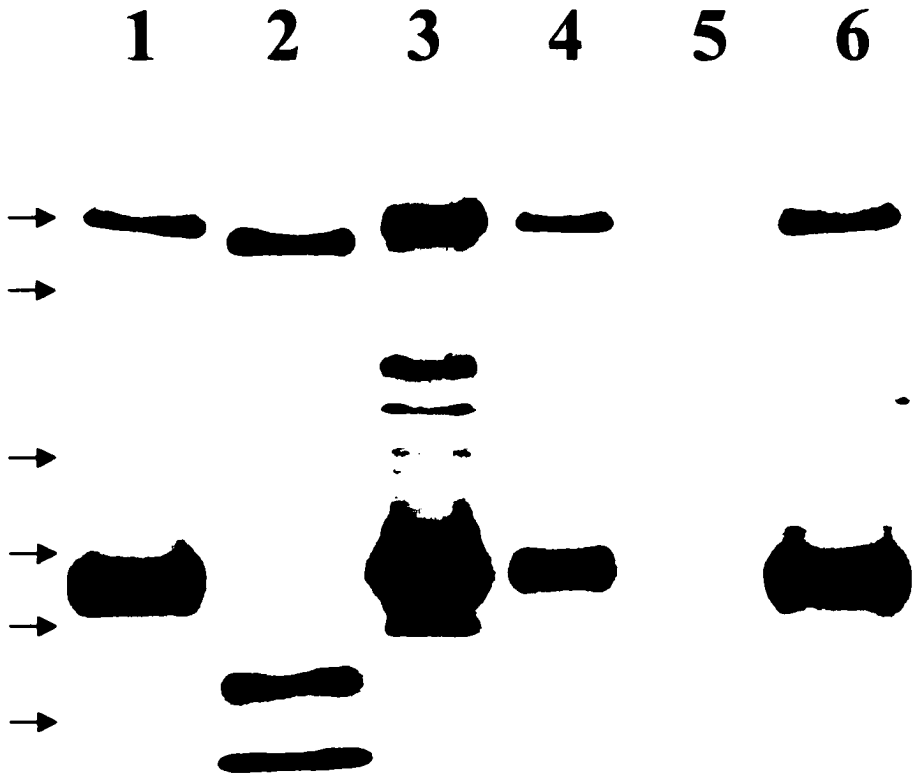


Figure 18. **Aerolysin binds to GPI-anchored proteins in brain including Thy-1 and a protein corresponding to contactin.** Mouse brain homogenate was incubated with or without PI-PLC and then centrifuged. Where indicated, a fraction of the supernatant from the PI-PLC-treated homogenate was incubated with PNGase F. Samples of resuspended pellets (20-30 μg protein/lane) and corresponding amounts of supernatants were separated by SDS-PAGE and blotted. Lane 1, supernatant from PI-PLC-treated homogenate, incubated under the same conditions as the supernatant in lane 2, but without PNGase F; lane 2, same supernatant as lane 1, treated with PNGase F; lane 3, untreated homogenate pellet control; lane 4, pellet from PI-PLC treated homogenate; lane 5, supernatant from untreated homogenate; lane 6, supernatant from PI-PLC treated homogenate. Arrows indicate positions of molecular weight markers. They are from the top 109, 80, 51.4, 34, 27, and 16.6 kDa.

migrate with apparent molecular masses around 110-kDa have been identified in brain tissue. These are 130-kDa contactin and 120-kDa NCAM (Mukasa *et al.*, 1995; Peles *et al.*, 1995). When deglycosylated the molecular masses of these proteins are approximately 100-kDa (contactin) and 80-kDa (NCAM; Nybroe *et al.*, 1989; Reid *et al.*, 1994). The change in molecular mass of the proaerolysin binding protein following deglycosylation was used to determine if it was more likely to be contactin or NCAM. When PI-PLC supernatant from brain homogenate was treated with peptide-N-glycosidase F, three major proaerolysin-binding bands appeared (Figure 18, lane 2). The lower band at 14.5-kDa corresponds to deglycosylated Thy-1, as seen before (Figure 7). The second band, migrating just above the 14.5-kDa band, either represents partially deglycosylated Thy-1 or another GPI-AP. The largest deglycosylated protein migrated around 95-kDa (Figure 18, lane 2), close to the mass of deglycosylated contactin (Reid *et al.*, 1994).

To unambiguously determine the identity of the 110-kDa protein, it was purified to homogeneity and the N-terminus was sequenced. Purification was accomplished by detergent solublization of a powder of chloroform:methanol extracted protein from brain, and subsequent lentil lectin and DEAE chromatography. This separated the unidentified 110-kDa protein from NCAM as revealed by immunoblotting with an antibody to NCAM (Figure 19B). A sandwich Western blot of the same samples revealed that proaerolysin only bound to the unidentified 110-kDa protein but not to NCAM (Figure 19C). The 110-kDa protein was then separated by SDS PAGE and blotted onto PVDF for N-terminal

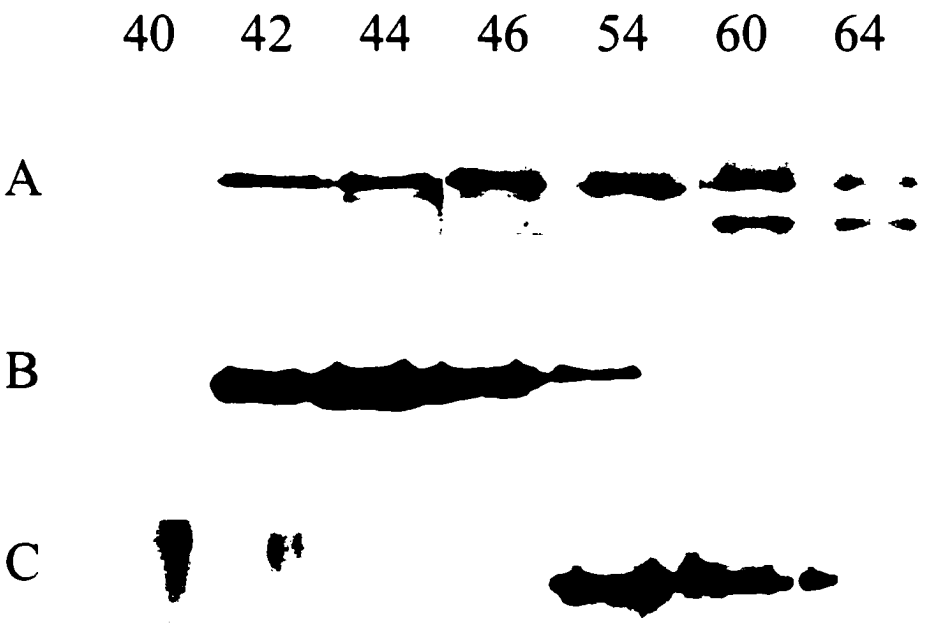


Figure 19. **Proaerolysin binds to contactin but not NCAM.** Samples of fractions off a DEAE column were silver stained (A) sandwich Western blotted (B) or blotted with an antibody to NCAM (C). Equal volumes of fractions were applied to each lane.

sequencing according to standard protocols. The resulting sequence (G-H-G-V-S-E-E-D-K-G-F-G-P-I-F-E-E-Q-P-I) corresponded to the N-terminal sequence of contactin (Peles *et al.*, 1995). Thus contactin is the 110-kDa proaerolysin-binding protein in brain.

Species specificity of proaerolysin binding

Having demonstrated that proaerolysin could bind to mouse and rat Thy-1 and contactin, I screened whole brain homogenates from several other species to determine if binding was species specific. The results in Figure 20 show that proaerolysin bound to proteins of similar size to Thy-1 and contactin and with similar intensity in human, mouse, pig, rabbit, and rat brain. Comparable binding was not observed in bovine or lamb brain, however it was not determined if this was because these species contain lower amounts of Thy-1 and contactin, or because the proteins differ in these species in some way that affects proaerolysin binding. These results suggest that proaerolysin-binding to Thy-1 and contactin is not strictly species specific.

Bovine and human erythrocytes were next screened for a proaerolysin receptor corresponding to the rat erythrocyte receptor EAR. A proaerolysin binding protein was identified on Western blots of membranes of erythrocytes from both species, migrating slightly higher than rat EAR (Figure 21 lanes 1, 3, and 5). Since EAR was previously shown to be glycosylated (Cowell *et al.*, 1997), it was possible that the difference in size between the three species was due to additional glycosylation of the human and bovine proteins. To test this possibility,

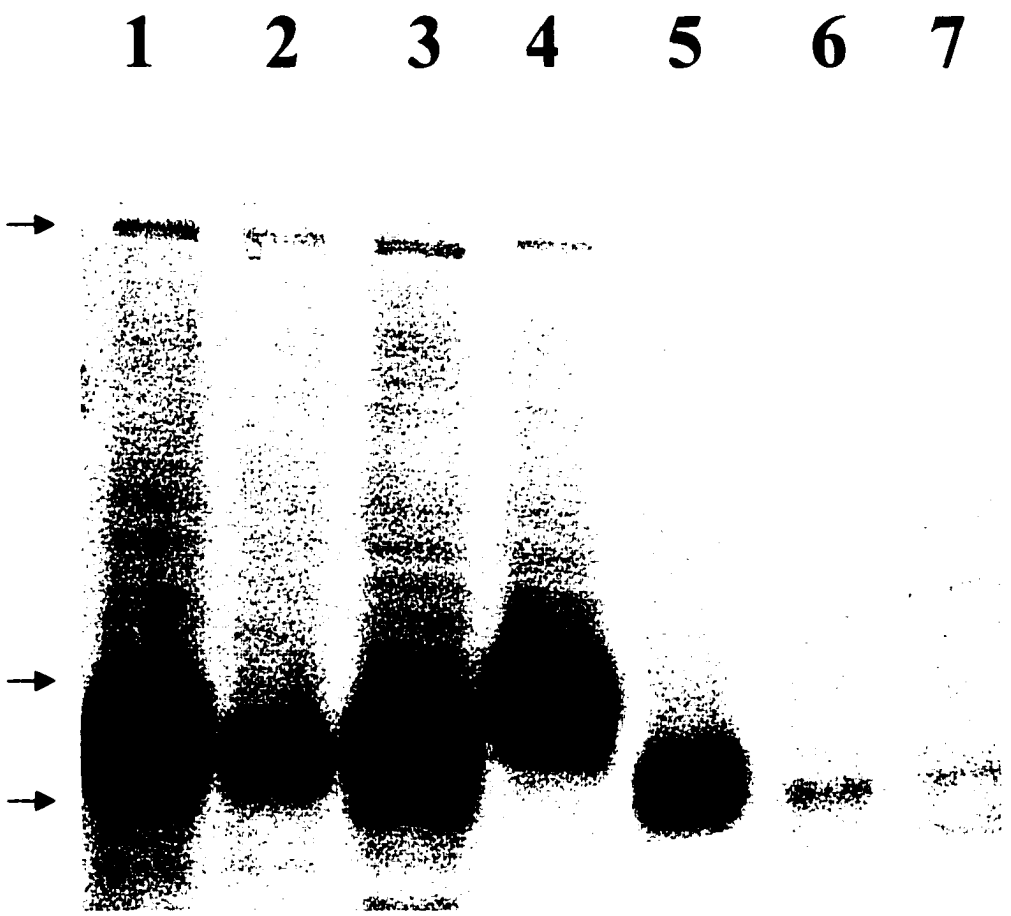


Figure 20. **Proaerolysin binding to blotted whole brain homogenates from different species.** Samples of brain from the following species containing approximately the same amount of protein (30 μg) was added to each lane. Lane 1, human; lane 2,mouse; lane 3, pig; lane 4, rabbit; lane 5, rat; lane 6, bovine; lane 7, lamb brain. Arrows indicate positions of molecular weight markers. They are from the top 109, 34, and 27-kDa.

Figure 21. **Bovine and human erythrocytes contain a proaerolysin-binding protein comparable to rat EAR.** Lanes 1 and 2 contain rat ghosts (6 μg of protein) incubated without (lane 1) or with PNGase F (lane 2); lanes 3, 4, 5, and 6 contain similarly treated bovine ghosts (10 μg of protein) and human ghosts (12 μg of proteins) respectively. Positions of the 51 and 34-kDa markers are indicated.

erythrocyte membranes were treated with peptide-N-glycosidase F. Deglycosylation revealed two proaerolysin binding bands of similar molecular masses in all three species (Figure 21, lanes 2, 4, and 6). The appearance of the two bands may indicate two GPI-APs common to all three species or it may be the result of partial deglycosylation of EAR by N-glycosidase, as mentioned previously (Cowell *et al.*, 1997). Whatever is the case, these results suggest that these erythrocytes share a related receptor.

To further demonstrate that the erythrocyte receptors were related I decided to investigate whether or not the human and bovine proteins were GPI-anchored. Previously, it was shown that rat EAR was released from membranes by PI-PLC treatment (Cowell *et al.*, 1997). To determine whether the bovine and human proteins were also GPI-anchored, samples of these membranes were treated with PI-PLC. The results in Figure 22 show that the bovine protein was released by the enzyme, whereas the human homologue was not. A distinguishing property of at least two human erythrocyte GPI-AP is that they remain attached to the membrane after treatment with PI-PLC (Brewis *et al.*, 1995; Rudd *et al.*, 1997). This is because an additional fatty acid on the inositol ring anchors the protein to the membrane after PI-PLC cleaves the phosphodiester bond linking the protein to the diacylglycerol anchor. Thus for human erythrocyte GPI-APs, the lack of release following PI-PLC treatment is not necessarily an indication that the protein is not GPI-anchored. Taken together these results indicate that all three species contain a related GPI-anchored proaerolysin-binding protein.

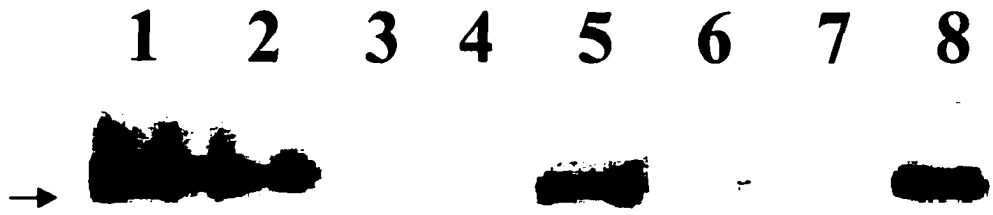


Figure 22. **Treatment with PI-PLC releases the bovine erythrocyte receptor from membranes, but not the human homologue.** Lane 1, untreated human ghosts; lane 2, PI-PLC-treated human ghosts; lane 3, supernatant from untreated ghosts; lane 4, supernatant from PI-PLC treated human ghosts. Lanes 5-8 contain corresponding samples from bovine ghosts. Approximately 20 μg of protein was applied in each lane containing ghosts, and corresponding amounts of supernatants were applied in the other lanes. The positions of the 51 kDa molecular weight marker is indicated.

The GPI-anchor itself is a binding determinant for proaerolysin

All of the proaerolysin-binding proteins so far identified are GPI-anchored. They include Thy-1 on T lymphomas and brain tissue, contactin from brain tissue, EAR on erythrocytes, placental alkaline phosphatase (PLAP), GP63 on CHO cells, and the trypanosomal variant surface glycoprotein (VSG; Figures 1, 18, 21, and 39; Nelson *et al.*, 1997, Diep *et al.*, 1998a). Since no significant sequence identity was apparent among these proteins, it seemed possible that the anchor itself was a binding determinant for proaerolysin (Diep *et al.*, 1998a). To investigate this possibility a variety of approaches were used. First, Dr. Srikumar Raja demonstrated that the removal of the entire GPI-anchor from VSG with hydrofluoric acid resulted in a decrease in proaerolysin binding to the protein, indicating that some portion of the anchor was required for optimal binding (Diep *et al.*, 1998a). Second, Dr. Dzung Diep demonstrated that the addition of a GPI-anchor to an otherwise soluble protein that was not bound by the toxin conferred proaerolysin binding ability on that protein. He also showed that Thy-1 expressed in *E. coli*, where it could not receive a GPI-anchor, was not bound by proaerolysin (Diep *et al.*, 1998a). These results demonstrated that the anchor is an important binding determinant for proaerolysin.

The anchor structure was next analyzed, to determine what portion was bound by proaerolysin. Since the acyl chains of GPI-anchors are embedded in the membrane they are unlikely to be accessible for binding. Evidence supporting this claim was provided by the fact that removal of the diacylglycerol moiety of the anchor of several GPI-APs with PI-PLC did not affect binding as determined by

sandwich Western blotting (Figures 7, 18 and 22). Additionally, proaerolysin seems to bind to proteins with and without acylated inositol (Figure 22). Not surprisingly, these results suggest that some portion of the GPI-anchor that is exposed on the membrane surface is bound by proaerolysin.

The structure of the GPI-anchor affects proaerolysin binding

Although GPI-anchors contain a conserved core, its modification leads to significant differences in anchor structure (Figure 4). For example, *Trypanosome brucei* procyclin, which is not bound by proaerolysin, has a highly modified anchor containing 23 additional sugar residues (Diep *et al.*, 1998a; Figure 23). In contrast, *Leishmania major* GP63 does not have any side chains added to the basic core anchor. It too is not bound by proaerolysin (Figure 23). These two proteins represent the two extremes in known anchor structure modification.

There is some evidence that GPI-anchors are species specific. Taking advantage of this, the ability of proaerolysin to bind a given GPI-AP expressed with either of two different GPI-anchors was next compared. The protein chosen for comparison was GP63 either present in cell lysates from *L. major* or expressed in CHO cells. The protein expressed in *L. major* is known to contain the basic core anchor, whereas the anchor of the protein expressed in CHO cells presumably contained an additional phosphoethanolamine residue on carbon two of the third mannose, as this is a common feature of mammalian GPI-anchors (Figure 24; Treumann *et al.*, 1998). This was likely the only difference between these two proteins as they were the same size based on migration in the gel and

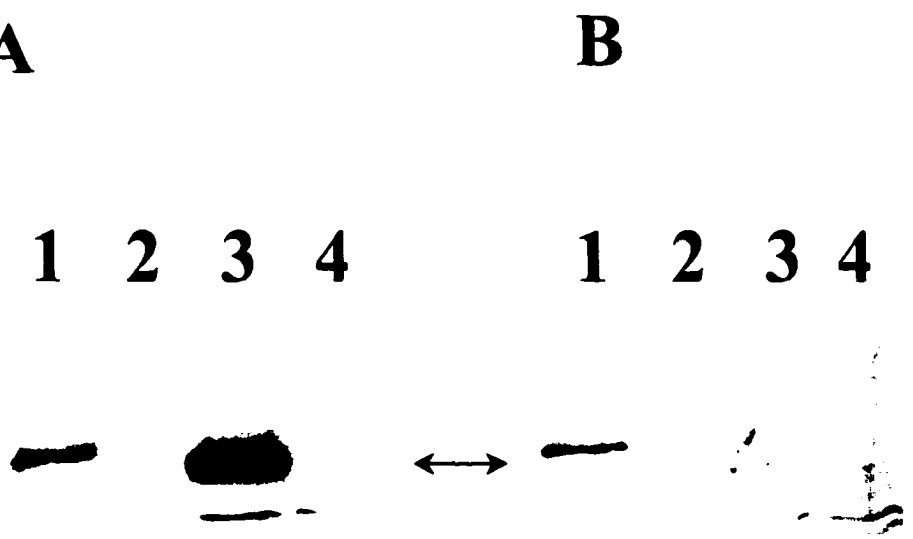
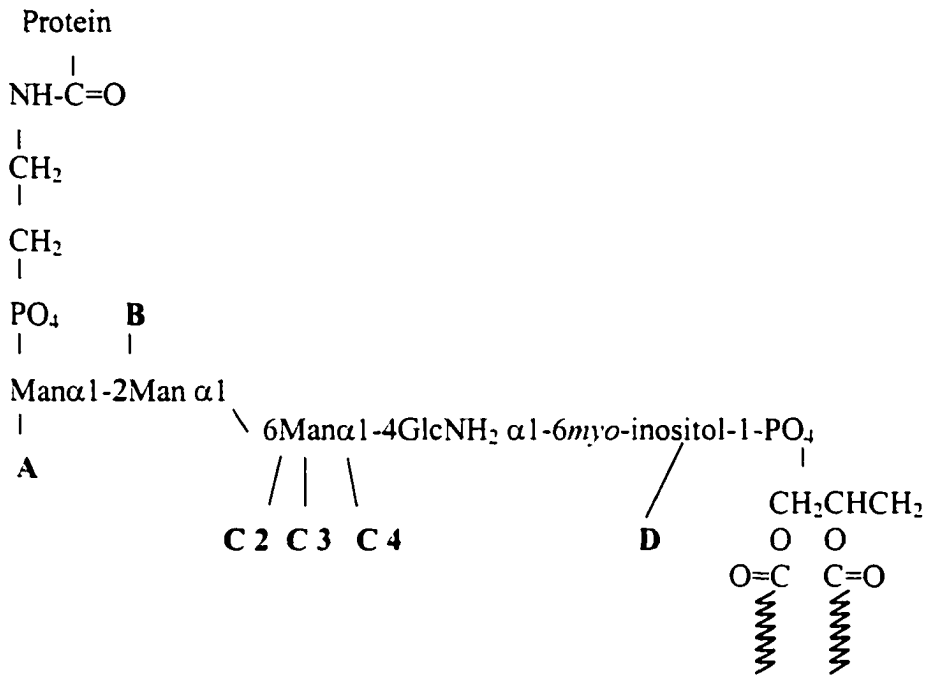


Figure 23. **Proaerolysin binds to gp63 expressed in CHO cells, but not to native gp63 expressed in *Leishmania major*.** Blots were developed with anti-gp63 (A) or with proaerolysin (B) as described in the methods. Equivalent amounts of cell lysates (10^5 cells) were applied to paired lanes. Lane 1, CHO cells expressing gp63; lane 2, control CHO cells; lane 3, wild type *Leishmania major*; lane 4, gp63 negative mutant strain. The double arrow marks the position of a 50.9 kDa standard.



Bound by toxin	Protein	A	B	C2	C3	C4	D
Yes	PLAP (human)	OH	+/- EtNP	EtNP	OH	OH	OH
Yes	Thy-1 (rat)	+/- Man α1-2	OH	EtNP	OH	GlcNAc β1-4	OH
Yes	VSG ? (trypanosome)	OH	OH	OH	+/- Galα1-2Galα1-6/ Galα1-3 +/- Galα1-2	OH	OH
No	GP63 (leishmania)	OH	OH	OH	OH	OH	OH
No	Procyclin (trypanosome)	$NANA_5$, $GlcNAc_9$, Gal_9					palm-itate

Figure 24. **A comparison of the anchor structures of proteins that are or are not recognized by proaerolysin.** The conserved core of all mammalian anchors contains an phosphoethanolamine (EtNP). Sites of modification of the third mannose at carbon 2, 3 or 4 (C2, 3 or 4) are indicated. The anchor structure commonly used by a variety of VSG is depicted here since the anchor structure of the VSG used in these studies is unknown.

both were recognized by the monoclonal anti-GP63 antibody (Figure 23B). Interestingly, the result showed that proaerolysin was only able to bind GP63 expressed in CHO cells (Figure 23B).

To obtain further information about the anchor structures recognized by proaerolysin, a comparison of the known GPI-anchored proaerolysin-binding proteins was performed. Six of eight tested mammalian proteins are bound by proaerolysin whereas only one of three parasite proteins is bound (Table 1; Figures 10, 18, 21, 23, 40; Diep *et al.*, 1998a). This suggests that there may be a fundamental difference in anchor structure between mammalian and parasite proteins that influences binding. Since all mammalian proteins contain an additional phosphoethanolamine residue, attached to carbon two of the third mannose (Figure 24), this residue plus the core anchor may form the minimum binding determinant for proaerolysin. The fact that some mammalian proteins are not recognized might be a result of the protein structure influencing binding, as will be discussed below. As mentioned above, one of the parasite proteins that is not recognized contains an unmodified anchor (GP63) whereas the other (procyclin) contains the most highly modified anchors identified to date (McConville and Ferguson, 1993). These two extremes in anchor structure may not be recognized as they do not contain the minimum structure found in mammalian proteins. The one parasite protein that is bound by proaerolysin is VSG. Although the anchor structure for the VSG used in these studies is not yet solved, by analogy with other forms of VSG it may be modified on the third mannose by addition of between one and four additional sugar residues

Protein Type	Protein	Bound by Proaerolysin
Mammalian Proteins	Cathepsin D in COS cells	Yes
	Contactin	Yes
	EAR	Yes
	GP63 in CHO cells	Yes
	PLAP	Yes
	Thy-1 (in brain, T lymphomas and CHO cells)	Yes
	Folate receptor in CHO cells	No
	NCAM	No
Parasite Proteins	GP63 from L. major	No
	Procyclin	No
	VSG	Yes

Table 1. **Binding of proaerolysin to various GPI-anchored proteins.**

(McConville and Ferguson, 1993). This structure shares similarities with the mammalian conserved core as both are modified at the third mannose (Figure 24). Further work will be required to determine how anchor structure influences proaerolysin binding.

A portion of the protein other than the GPI-anchor is required for proaerolysin binding

Although the GPI-anchor is clearly a binding determinant for proaerolysin, some part of the protein also appears to influence binding. This is demonstrated by the fact that although GPI-APs expressed on a given cell type likely have the same anchor, proaerolysin does not appear to bind to all of them. For example, proaerolysin was able to detect purified contactin whereas NCAM from the same cell type was not recognized (Figures 18 and 19C). In addition, proaerolysin was able to bind GP63 and Thy-1 expressed in CHO cells, whereas the folate receptor expressed in the same cell line was not bound (Diep *et al.*, 1998a; Gordon *et al.*, 1999; Figure 23). These results demonstrate that the GPI-anchor alone is not sufficient for proaerolysin binding.

Aerolysin induces apoptosis of T lymphomas

Low concentrations of aerolysin trigger apoptosis of T lymphomas

Following binding, aerolysin oligomerizes in order to form heptamers and this may result in clustering of GPI-APs. Both binding and cross-linking of GPI-APs have been shown to induce apoptosis in T lymphomas (Hueber *et al.*, 1994).

In addition, channel formation by several bacterial pore-forming toxins has been shown to trigger apoptosis (Chen and Zychlinsky, 1994; Muller *et al.*, 1999). To determine whether or not this was also true for aerolysin, EL4 cells were incubated overnight with a range of proaerolysin concentrations, and then analyzed for two classical signs of apoptosis; DNA fragmentation, and increased caspase-3 activity. Both were observed in cells treated with 10^{-10} M proaerolysin, evidence that apoptosis occurs at this toxin concentration, but were less apparent in cells treated with higher or lower proaerolysin concentrations (Figure 25A and B).

Characterization of proaerolysin variants used in apoptosis studies

As mentioned above, apoptosis could result from binding to GPI-APs, clustering of GPI-APs, or channel formation. To distinguish between these three possibilities, aerolysin variants deficient in oligomerization (T253C/A300C) or channel formation (Y221G) were used. To demonstrate that the proaerolysin variant T253C/A300C is unable to oligomerize, erythrocytes were incubated with 10^{-8} M wild-type, T253C, A300C, or T253C/A300C proaerolysin or aerolysin, and then incubated at 37°C to allow for oligomerization. Samples of cells were then washed, suspended in sample buffer, separated by SDS PAGE and blotted to screen for the oligomer. The results in Figure 26 demonstrate that wild-type and the single mutants T253C and A300C can oligomerize, but that the double mutant T253C/A300C cannot. This is because a disulfide bridge forms between

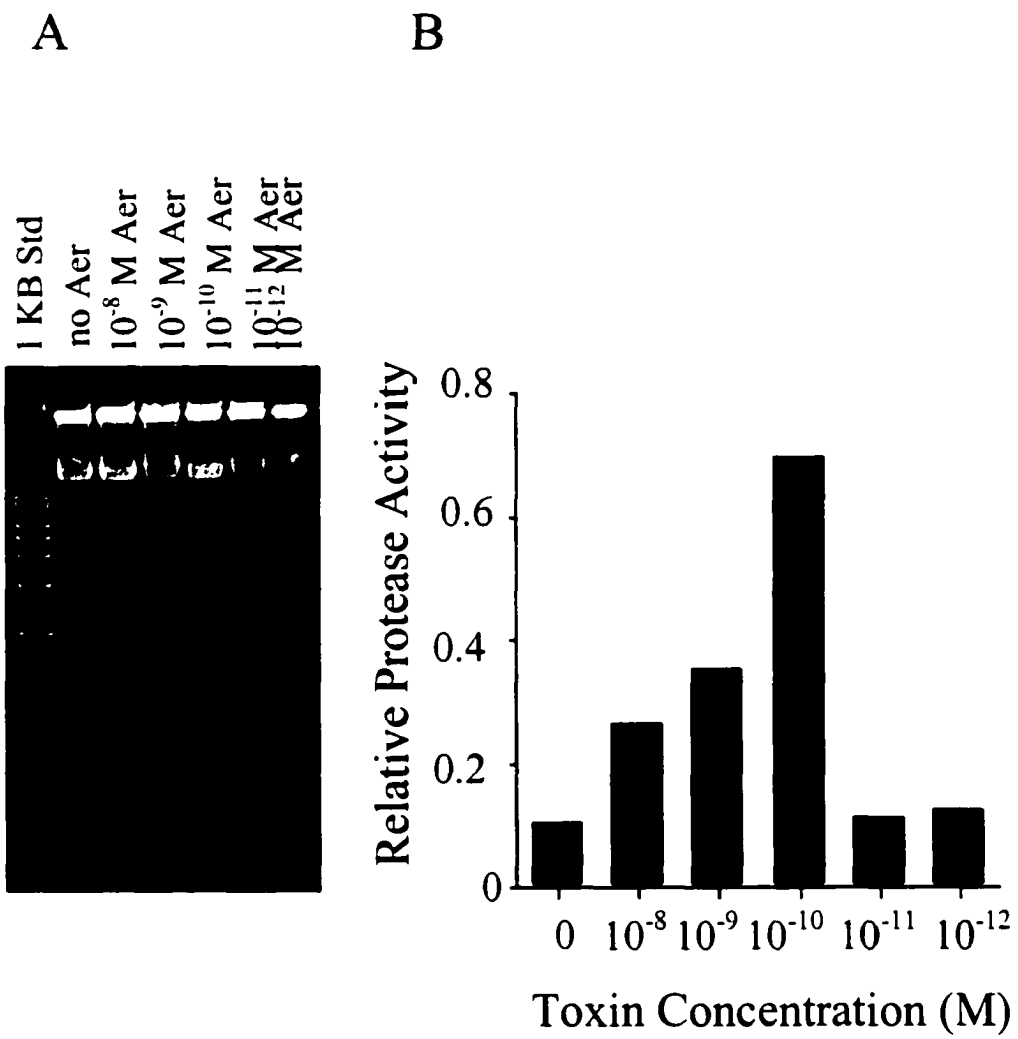


Figure 25. **Aerolysin induces apoptosis in T lymphomas at low toxin concentrations.** A) Electrophoretic analysis of DNA fragmentation in EL4 T lymphomas treated with and without indicated aerolysin concentrations. B) Caspase-3 activity of EL4 T lymphomas treated with the same toxin concentrations as in A.

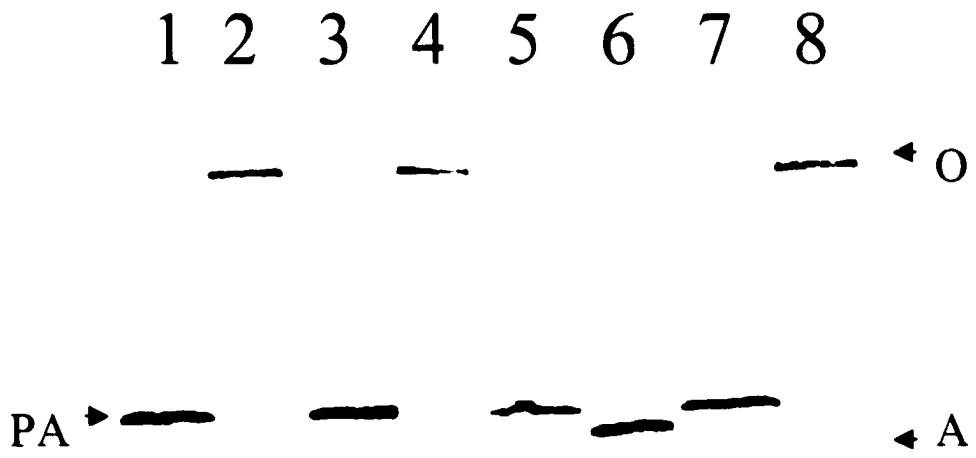


Figure 26. **Binding and oligomerization of cysteine mutants on rat erythrocytes.** Rat erythrocytes were incubated with the proaerolysin or aerolysin forms of wild-type or cysteine mutants. The erythrocyte membranes were then isolated, suspended in SDS-PAGE sample buffer, and separated by SDS-PAGE and blotted. Proaerolysin (PA), aerolysin (A) and oligomer (O) bound to the membranes was detected with polyclonal anti-aerolysin followed by anti-rabbit HRPO. Lanes 1 and 2 contain samples of erythrocytes exposed to wildtype proaerolysin (lane 1), and aerolysin (lane 2); lanes 3 and 4, T253C proaerolysin, and aerolysin; lanes 5 and 6, T253C/A300C proaerolysin and aerolysin; and lanes 7 and 8, A300C proaerolysin and aerolysin.

253C and 300C that prevents a loop that normally shifts during oligomerization from moving thus inhibiting oligomerization (Rossjohn *et al.*, 1998).

The proaerolysin variant Y221G is blocked at a later stage in the aerolysin pathway. It can oligomerize but it appears to be defective in insertion or in forming open channels (unpublished data). To demonstrate that Y221G could oligomerize just as well as wild-type aerolysin, erythrocytes were incubated with 10^{-8}, 10^{-9} and 10^{-10} M wild-type or Y221G aerolysin as above and then blotted (Figure 27).

To determine what effect these variants were having on EL4 cells they were labeled with Alexa 488 along with wild-type proaerolysin and then used to label cells. Their distribution on the cell surface was then compared. At 4°C, wild-type aerolysin and both variants appeared evenly distributed on the surface of the cell (Figure 28). When the cells were warmed to 37°C, so that the proteins could become mobile on the surface, and have the opportunity to oligomerize, different effects were observed. The results in Figure 28 show that exposure to wild-type aerolysin resulted in punctate staining and disrupted cells (Figure 28; small arrow). This was evidence that it was oligomerizing and forming channels in the plasma membrane. In contrast, there was no significant change in the distribution of FLAER T253C/A300C at 37°C, indicating that as expected this variant was not oligomerizing and causing clustering of receptors, but was only binding to them. In contrast, there was a significant change in the distribution of FLAER Y221G at 37°C, which was characterized by the presence of bright caps on the poles of cells (Figure 28; large arrowhead). When proteins are crosslinked

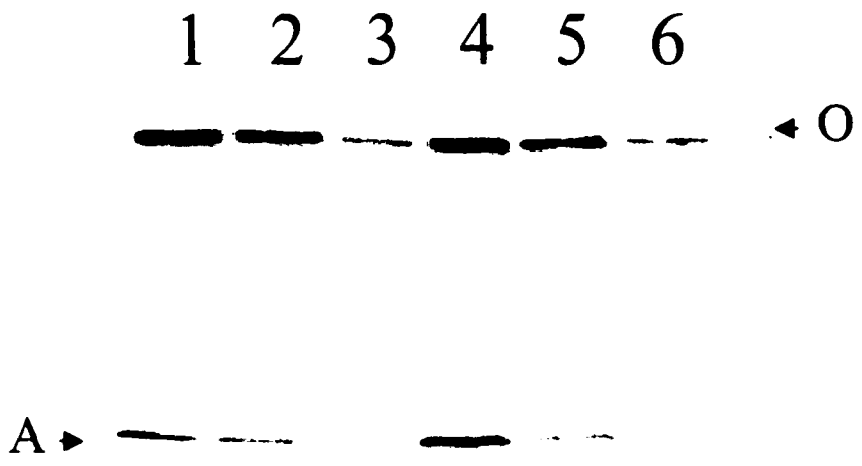


Figure 27. **Binding and oligomerization of aerolysin variant Y221G.** Rat erythrocytes were incubated with 5 x 10^{-8}, 5 x 10^{-9}, and 5 x 10^{-10} M wild-type aerolysin (lanes 1-3 respectively) or aerolysin variant Y221G (lanes 4-6). The erythrocyte membranes were then isolated, suspended in SDS-PAGE sample buffer, and separated by SDS-PAGE and blotted. Aerolysin (A) and oligomer (O) bound to the membranes was detected with polyclonal anti-aerolysin followed by anti-rabbit HRPO.

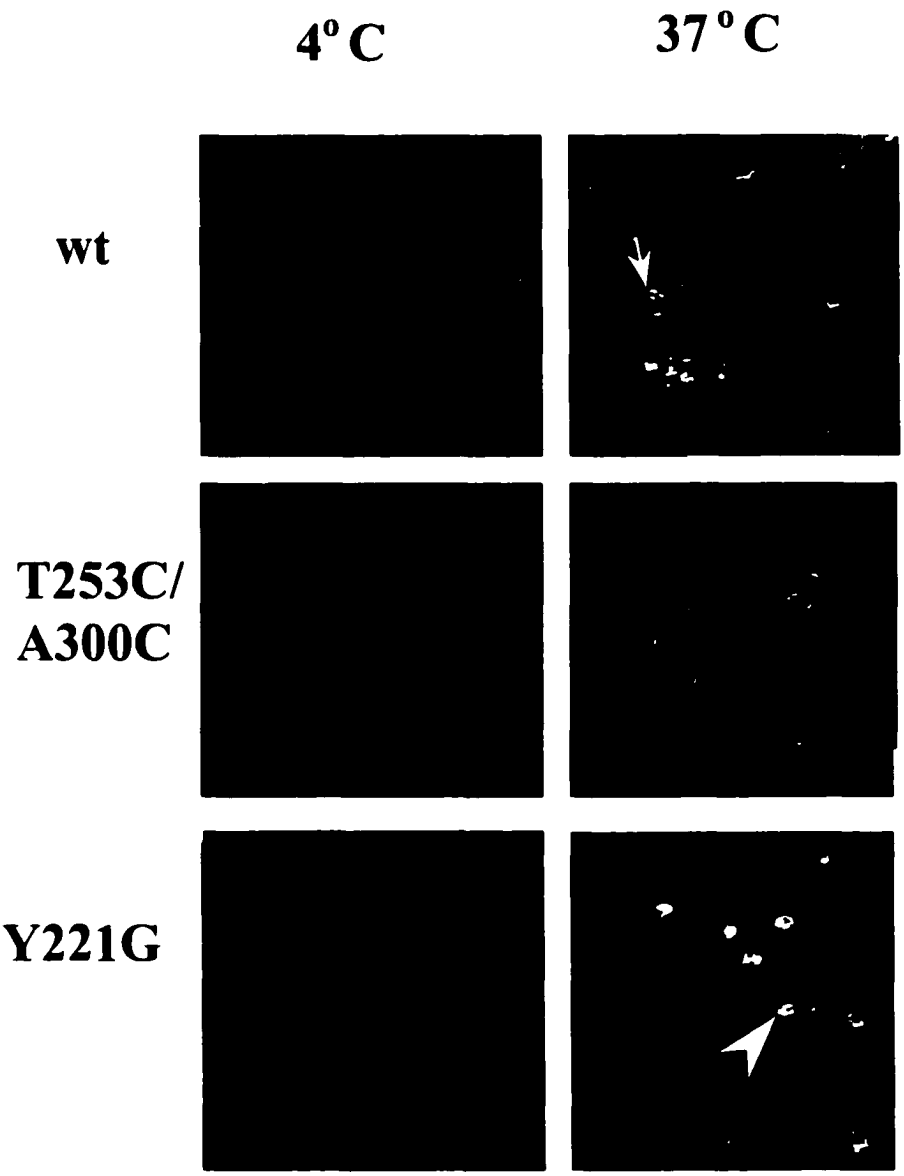


Figure 28. **Binding and localization of wt, Y221G, and T253C/A300C aerolysin on the surface of EL4 lymphomas as visualized by confocal microscopy**. Cells were incubated with 10^{-8} M indicated FLAER variants on ice. Following labeling cells were washed and either left on ice or treated at 37 °C for 30 minutes. Cells were then fixed and visualized by confocal microscopy as described. Small arrows indicate the position of clustered wild-type aerolysin and large arrowheads indicate the position of capped Y221G aerolysin.

on the surface of T lymphocytes, a process known as capping occurs, in which the cell actively sweeps the crosslinked proteins to one pole of the cell (Dellagi and Brouet, 1982; Kammer *et al.*, 1988). The capping observed with FLAER Y221G is presumably the result of oligomerization causing crosslinking of aerolysin on the cell surface.

Ability of aerolysin variants to induce apoptosis

Having demonstrated that the proaerolysin variant T253C/A300C is only able to bind to receptors, while the variant Y221G can also cause receptor clustering, their ability to induce apoptosis once activated was compared to native proaerolysin, which induces apoptosis at 10^{-10} M. The results in Figure 29 show that the aerolysin variant (T253C/A300C) had little or no effect on the cells at any concentration below approximately 10^{-5} M, indicating that receptor binding was not enough to trigger apoptosis. The variant Y221G also was unable to induce apoptosis at wild-type concentrations (Figure 29), even though large amounts of oligomer could form (Figure 27). Thus it does not appear that aerolysin-induced apoptosis is the result of binding or clustering of GPI-anchored receptors.

Glycosylphosphatidylinositol-anchored proteins are not required to trigger aerolysin-induced apoptosis

To completely rule out the possibility that GPI-APs were required for aerolysin-induced apoptosis, cells lacking GPI-APs (EL4 (Thy-1-f)) were treated with a range of proaerolysin concentrations and then screened for indicators of

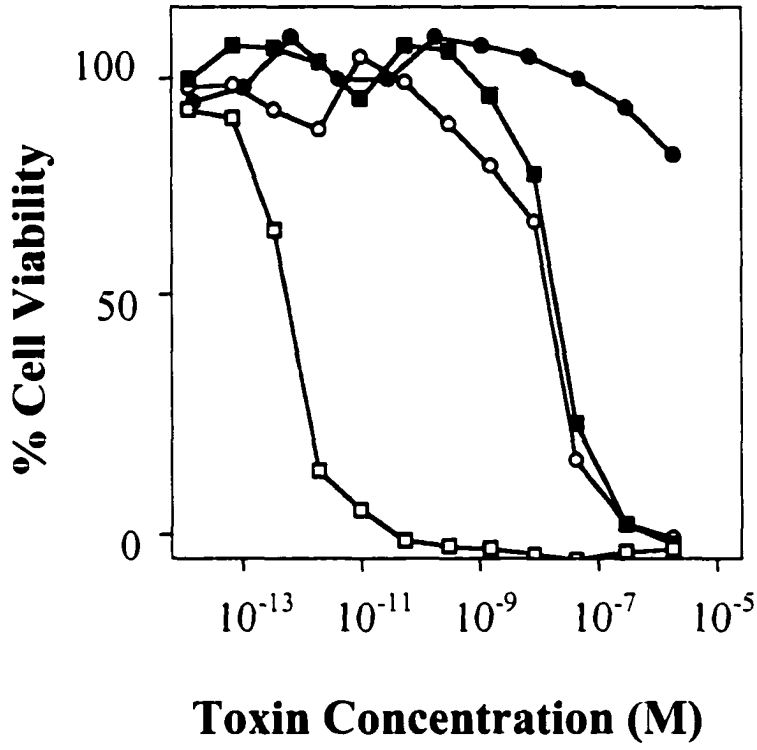


Figure 29. **Effects of proaerolysin and proaerolysin variants on T lymphomas.** Cells were treated with indicated toxin concentrations for 18.5 hours. Cell viability was measured using the MTS/PMS CellTiter96 cell viability assay from Promega. Wild-type proaerolysin and EL4 cells (□); wild-type proaerolysin and EL4 (Thy-1-f) cells (■); Y221G proaerolysin and EL4 cells (o); T253C/A300C proaerolysin and EL4 cells (●).

apoptosis. Both DNA fragmentation and an increase in caspase-3 activity were observed in these cells, although much higher concentrations of proaerolysin were required, indicating apoptosis can occur in the absence of GPI-APs (Figure 30). The difference in toxin concentration required to induce apoptosis in EL4 cells (10^{-10} M) *versus* EL4 (Thy-1-f) cells (10^{-6} to 10^{-7} M) is presumably a reflection of the higher concentration of aerolysin required to cause channel formation in the absence of GPI-anchored receptors (see below).

Channel formation occurs at concentrations where apoptosis is observed

Since my results indicated GPI-APs were not directly involved in aerolysin-induced apoptosis, it seemed likely that channel formation was triggering apoptosis. The generation of a small number of channels by other pore-forming proteins has previously been shown to induce apoptosis (Chen and Zychlinsky, 1994; Muller *et al.*, 1999). A rise in intracellular calcium, which has been suggested to be the trigger for channel-induced apoptosis (McConkey *et al.*, 1988; Martikainen *et al.*, 1991; Muller *et al.*, 1999), occurs as calcium, among other ions, passes through channels formed in the plasma membrane from the external medium. To look for calcium influx at the concentration of proaerolysin where apoptosis was observed, cells were loaded with the fluorescent calcium indicator Fluo3-AM, and then exposed to different concentrations of aerolysin and analyzed by flow cytometry over time. The results in Figure 31 show that treatment of EL4 cells with aerolysin concentrations as low as 10^{-10} M caused maximal increases in intracellular calcium as measured by an increase in

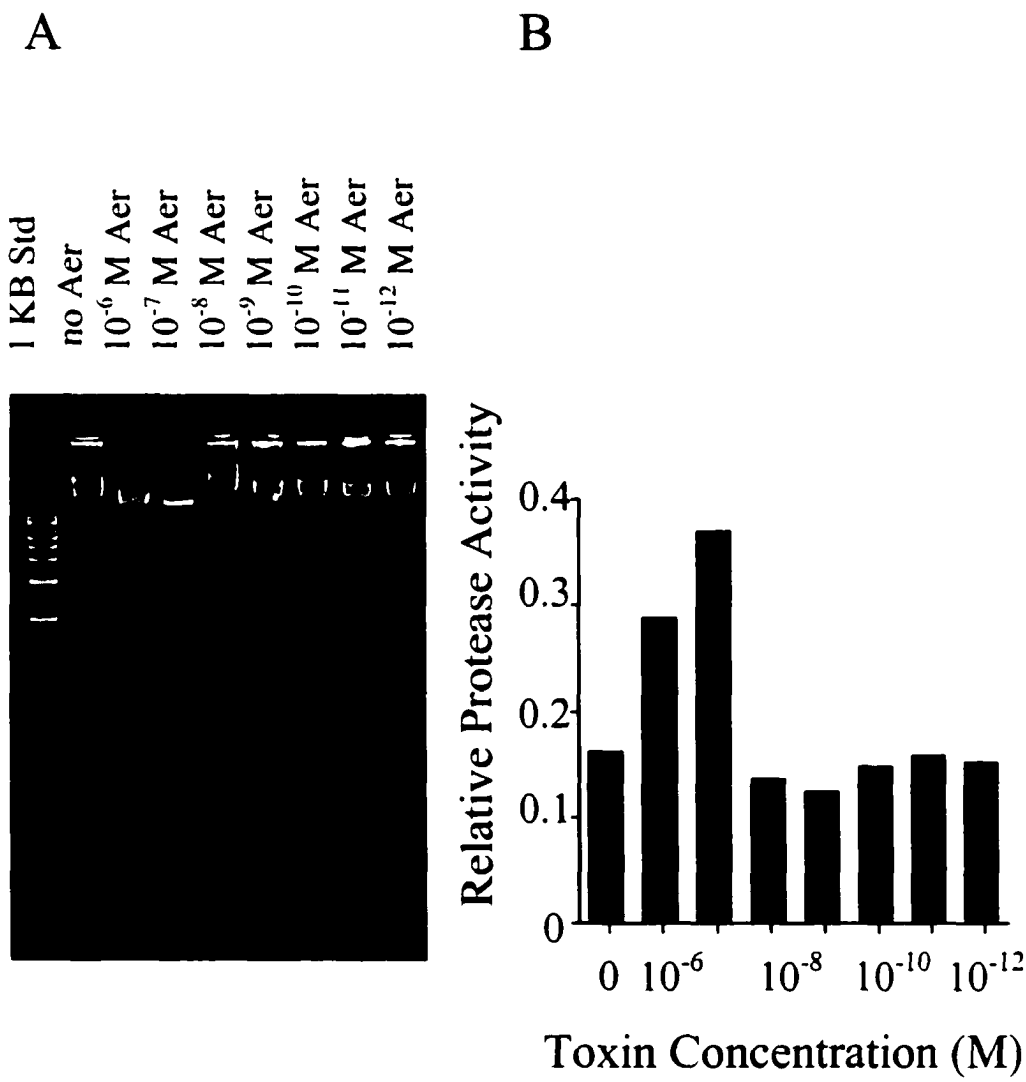


Figure 30. **Aerolysin induces apoptosis in T lymphomas lacking GPI-anchored proteins.** A) Electrophoretic analysis of DNA fragmentation in EL4 (Thy-1-f) lymphomas treated with and without indicated aerolysin concentrations. B) Caspase-3 activity of EL4 (Thy-1-f) lymphomas treated with the same toxin concentrations as in A.

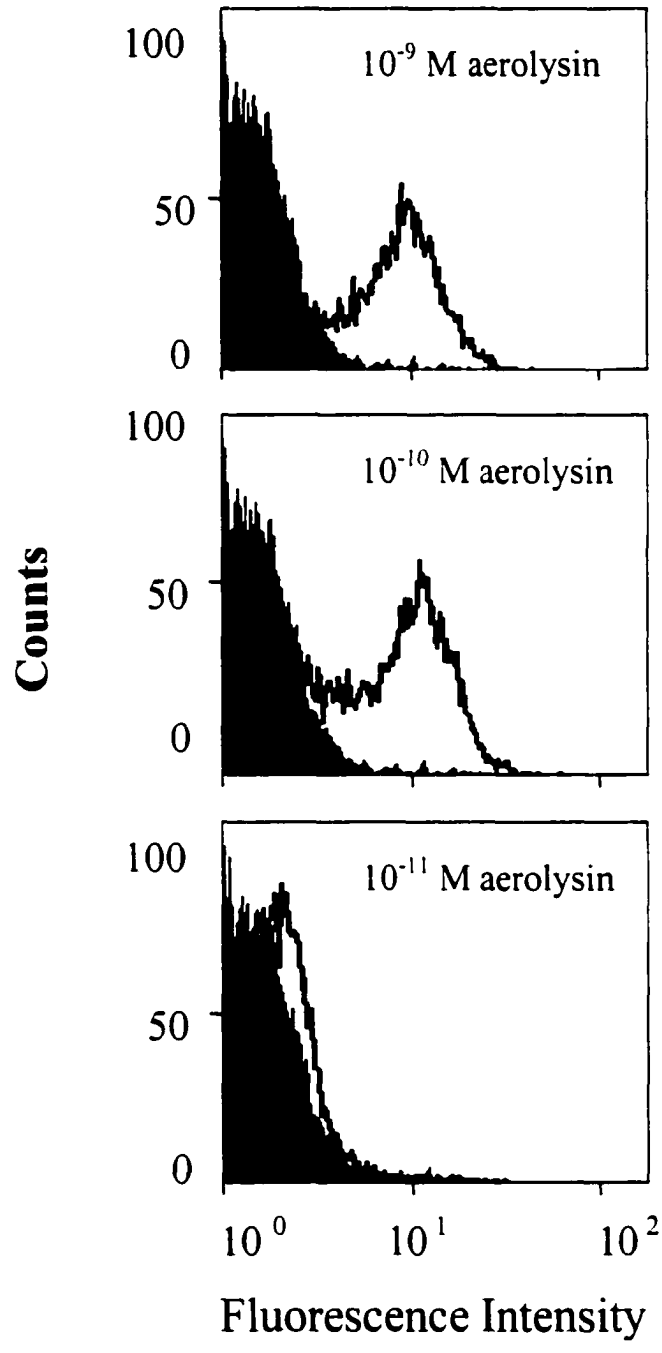


Figure 31. **Channel formation occurs at concentrations where apoptosis is observed in T lymphomas.** EL4 cells were loaded with the calcium indicator Fluo3-AM and analyzed by flow cytometry following treatment with 10^{-9} M, 10^{-10} M, or 10^{-11}M proaerolysin (unfilled profile) or without proaerolysin (grey profile) for 30 minutes.

fluorescence within 30 minutes. To demonstrate that this increase was due to the influx of cations from the medium and not a result of the release of calcium from intracellular stores, experiments were carried out in medium lacking calcium. The results in Figure 32 demonstrate that there is no increase if cells are incubated in calcium free medium. These results demonstrate that channel formation accompanied by an influx of calcium from the medium is occurring at concentrations where apoptosis is observed.

Aerolysin activity is not promoted by lipid raft domains

Recently it was suggested that lipid rafts play an important role in channel formation by aerolysin by concentrating the toxin on the cell surface and promoting its oligomerization (Abrami and van der Goot, 1999). However, although these authors showed that proaerolysin was associated with rafts, they did not determine whether this association had any measurable effect on aerolysin activity. The experiments described here were designed to investigate whether or not raft domains play any role in channel formation.

Some proaerolysin is associated with a detergent insoluble fraction

A characteristic feature of lipid rafts is their insolubility in some cold non-ionic detergents (Fra *et al.*, 1994; Schroeder *et al.*, 1994). Due to their high lipid content, they have a low density and they can be separated from other cell components by flotation in sucrose density gradients (Fra *et al.*, 1994). Since GPI-

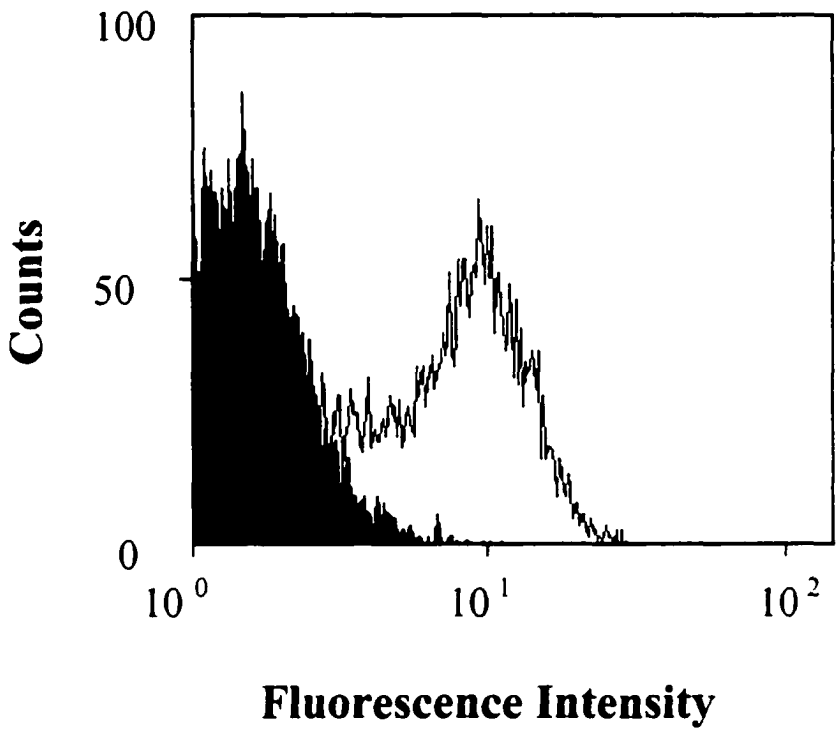


Figure 32. **The increase in intracellular calcium observed in cells treated with 10^{-10} M aerolysin is caused by calcium influx from the media not by mobilization of intracellular calcium stores.** EL4 cells were loaded with the calcium indicator Fluo3-AM and then treated with aerolysin for 30 minutes in normal medium (unfilled profile) or calcium-free medium (filled profile).

APs appear to be transiently localized to raft domains (Sheets *et al.*, 1997), they are enriched in the floating raft fraction following detergent extraction (Benting *et al.*, 1999; Mayor and Maxfield, 1995). Proaerolysin binds with high affinity to GPI-APs, so it too should be found in the raft fraction after detergent extraction of toxin labeled cells. This association was first reported after detergent extraction of baby hamster kidney cells by Abrami and van der Goot who presented evidence that appeared to show that most bound aerolysin was associated with rafts (1999). In contrast, I found that only a fraction of the proaerolysin on the surface of EL4 cells associated with raft domains (Figure 33A, lanes 2 and 3), while the rest of the protoxin was distributed in non-raft domains (Figure 33A, lanes 8-11 and pellet). This is consistent with the fact that only a fraction of cell surface GPI-APs are located in raft domains (Kenworthy *et al.*, 1998; Kenworthy and Edidin, 2000). A direct comparison of the results presented here with the results obtained by Abrami and van der Goot (1999) cannot be made, since these authors chose to compare fractions on their SDS-PAGE gels based on constant total protein instead of equal volume, thereby heavily weighting the apparent amount of proaerolysin found in the raft fraction.

The fact that a fraction of the surface bound toxin was found to associate with the detergent insoluble fraction is not by itself evidence that there is a physiologically significant association of the toxin with rafts *in situ*. The results in Figure 33B show that proaerolysin was still able to associate with the raft fraction even if it was added after the cells had been disrupted with Triton X-100. The amount of association was comparable to the amount of raft-associated toxin from

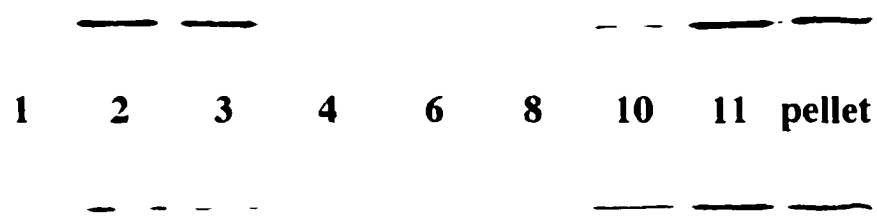


Figure 33. **Proaerolysin is partly recovered in a detergent-insoluble fraction when added before or after cell disruption with Triton X-100.** Top panel, EL4 cells were incubated with 10^{-8} M Y221G and then extracted with Triton X-100 as described in the text; or were extracted with detergent and then Y221G proaerolysin was added to 10^{-8} M (bottom panel). Following sucrose gradient centrifugation, 1 ml fractions were collected from the top of the tube and analyzed for proaerolysin by Western blotting. Numbers correspond to fractions off the gradient.

cells that had been preincubated with the toxin before extraction (compare Figures 33A and 33B). Thus the recovery of proaerolysin in a raft fraction is not evidence that the toxin actually associates with rafts *in situ*.

Raft disruption does not affect channel formation in T lymphomas

I next wanted to determine whether or not the presence of rafts increases cell sensitivity to aerolysin as implied by Abrami and van der Goot (1999). Since cholesterol is a critical structural component of lipid rafts, lowering its concentration by treating cells with the cholesterol sequestering agent methyl-β-cyclodextrin results in their disruption (Ilangumaran *et al.*, 1998). Extraction of EL4 cells with 10 mM methyl-β-cyclodextrin for 30 minutes led to a 45 % decrease in cholesterol levels. Evidence that rafts had been disrupted was provided by the fact that there was a decrease in the amount of Thy-1 in the detergent insoluble fraction (Figure 34). To ensure that cholesterol extraction was not affecting proaerolysin binding, binding of FLAER Y221G to treated and untreated cells was compared by flow cytometry. The results in Figure 35 show that cholesterol extraction had little or no effect on proaerolysin binding. This was not surprising, since proaerolysin should bind to GPI-APs no matter where they are localized on the cell surface. I next compared the sensitivity of the normal cells and the methyl-β-cyclodextrin treated cells to proaerolysin. The results in Figure 36 indicate that raft disruption had no effect on the sensitivity of the cells to aerolysin.

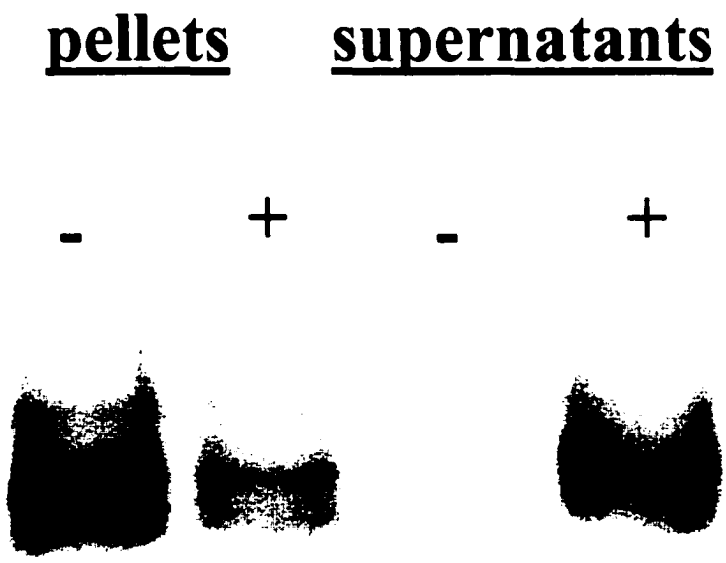


Figure 34. **Cholesterol depletion results in a decrease in the amount of Thy-1 associated with rafts.** Following methyl-β-cyclodextrin extraction the distribution of GPI-anchored proteins on T lymphomas was analyzed by Triton X-100 extraction as described in the text. Thy-1 was detected by sandwich Western blotting.

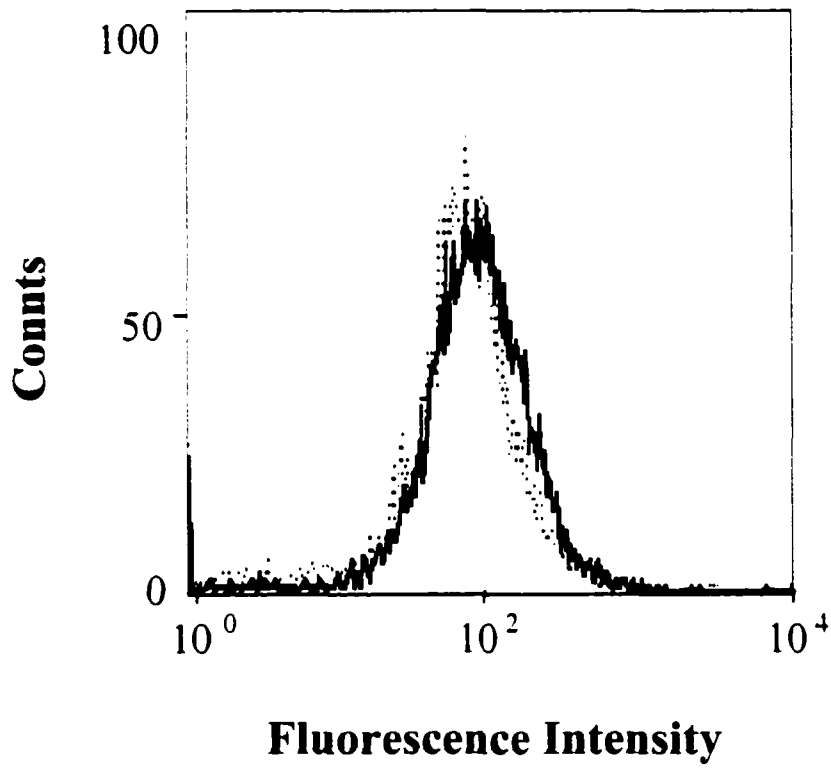


Figure 35. **Raft reduction has no effect on the binding of aerolysin to lymphomas.** FACS analysis of binding of fluorescently labeled aerolysin (FLAER) to lymphomas before (solid line) and after (dotted line) following methyl-β-cyclodextrin extraction. Samples were labeled as described in the methods.

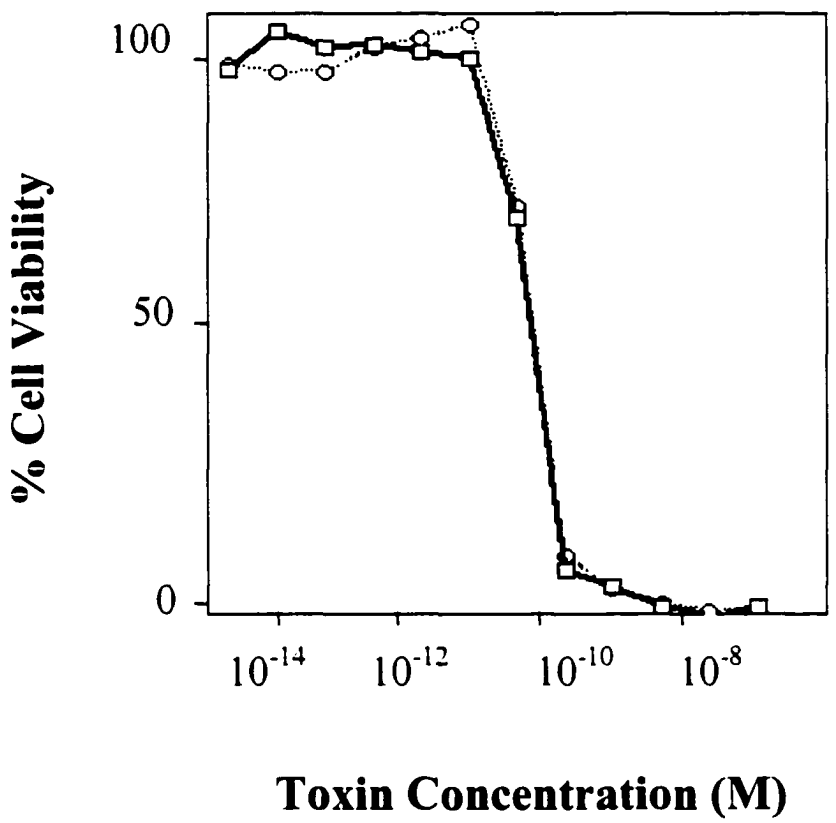


Figure 36. **Raft reduction has no effect on the sensitivity of T lymphomas to aerolysin.** Sensitivity of T lymphomas to aerolysin before (□) and after (o) raft disruption with methyl-β-cyclodextrin. Cell viability was measured with an MTS/PMS cell viability assay as described in the text.

Cholesterol depletion does not decrease the sensitivity of erythrocytes to aerolysin

To ensure that the lack of an effect on cell proaerolysin sensitivity observed following raft disruption was not a unique property of EL4 cells or the result of incomplete raft disruption, experiments were also carried out with erythrocytes. Erythrocytes and T lymphomas are equally sensitive to aerolysin (Figure 37), even though they contain different GPI-anchored aerolysin binding proteins. This was expected since aerolysin uses the GPI-anchor as a binding determinant, which contains a core structure that is conserved between cells types and species (Brewis *et al.*, 1995; Diep *et al.*, 1998a; Nelson *et al.*, 1997). Treatment of erythrocytes with 3.5 mM cyclodextrin for 1 hour resulted in a 90 % reduction in total cell cholesterol. The higher percentage of cholesterol reduction obtained with erythrocytes is likely due to the fact that all of the cholesterol is plasma membrane associated, and therefore accessible to the sequestrant. The results in Figure 38 show that this nearly complete cholesterol depletion did not decrease the rate of channel formation by aerolysin, even though the fraction of GPI-APs associated with raft domains must have been very small or negligible.

Rafts domains on liposomes do not promote channel formation

The above results indicated that lipid rafts do not promote channel formation by aerolysin in T lymphomas and erythrocytes, contrary to the prediction of Abrami and van der Goot (1999). However, although cyclodextrin

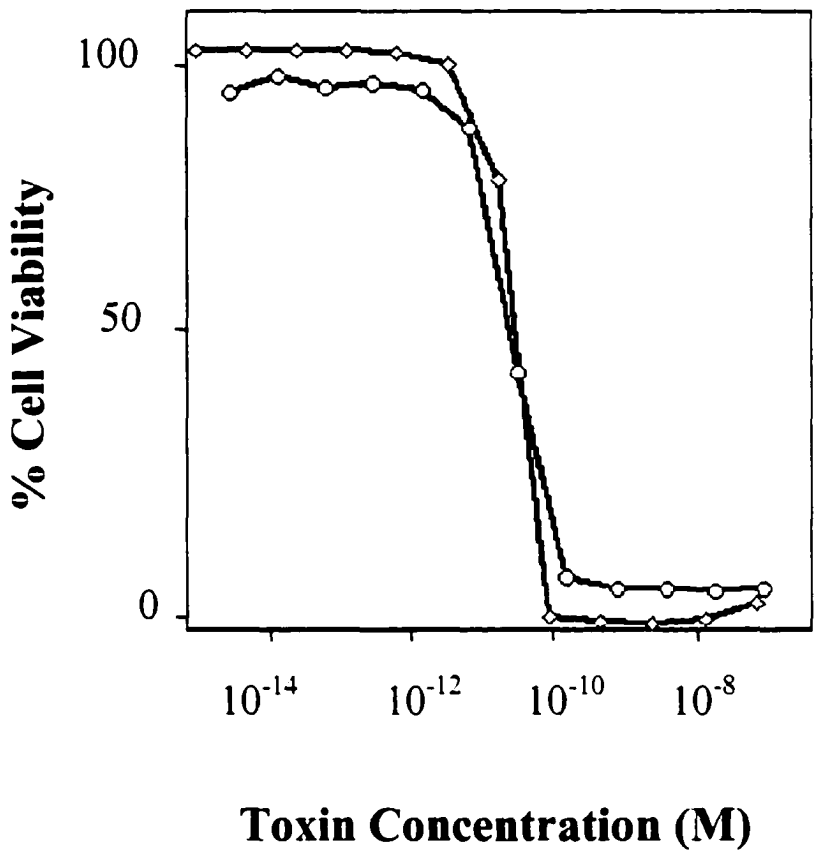


Figure 37. **Erythrocytes and T lymphomas are equally sensitive to aerolysin.** The sensitivity of T lymphomas (o) and rat erythrocytes (◇) to aerolysin was compared as described.

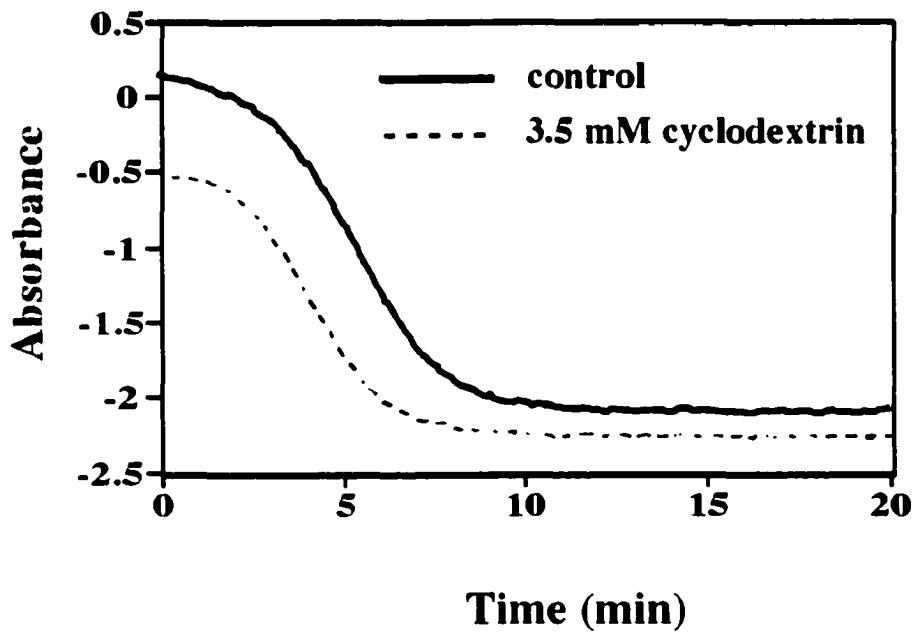


Figure 38. **Erythrocytes do not become less sensitive to aerolysin following cholesterol extraction.** Kinetic analysis of aerolysin-induced hemolysis of erythrocytes treated with no (solid line), or 3.5 mM methyl-β-cyclodextrin (dotted line).

treatment is a well characterized method for disrupting rafts, I could not be certain that all raft domains had been disrupted, even in the erythrocytes. In order to compare the sensitivity of membranes with and completely without raft domains to aerolysin, large unilamellar liposomes containing incorporated aerolysin receptors were used. Liposomes without rafts were made from phospholipids and cholesterol, whereas liposomes with rafts were made from lipid mixtures containing sphingomyelin. The GPI-AP Thy-1 had previously been incorporated into liposomes in experiments that showed that it acts as a receptor for aerolysin (Nelson *et al.*, 1997). To monitor channel formation the liposomes were loaded with carboxyfluorescein (CF), a self-quenching molecule whose fluorescence increases upon dilution. Channel formation by aerolysin allows CF to leak out of the liposomes and the dilution leads to an increase in fluorescence that is monitored over time by spectrofluorometry (Nelson *et al.*, 1997). I used a similar system to compare the sensitivity of liposomes with and without rafts to aerolysin. For these studies, Thy-1 was replaced with the GPI-anchored enzyme placental alkaline phosphatase (PLAP) because the presence of PLAP can easily be quantitated by measuring its enzymatic activity, and used to match the amount of receptor in each liposome population (Nelson *et al.*, 2000). The results in Figure 39A show that liposomes containing GPI-anchored PLAP were much more sensitive to aerolysin than those without PLAP, as we had previously observed with Thy-1 (Nelson *et al.*, 1997). This indicates that PLAP is yet another GPI-AP that can function as a receptor for aerolysin.

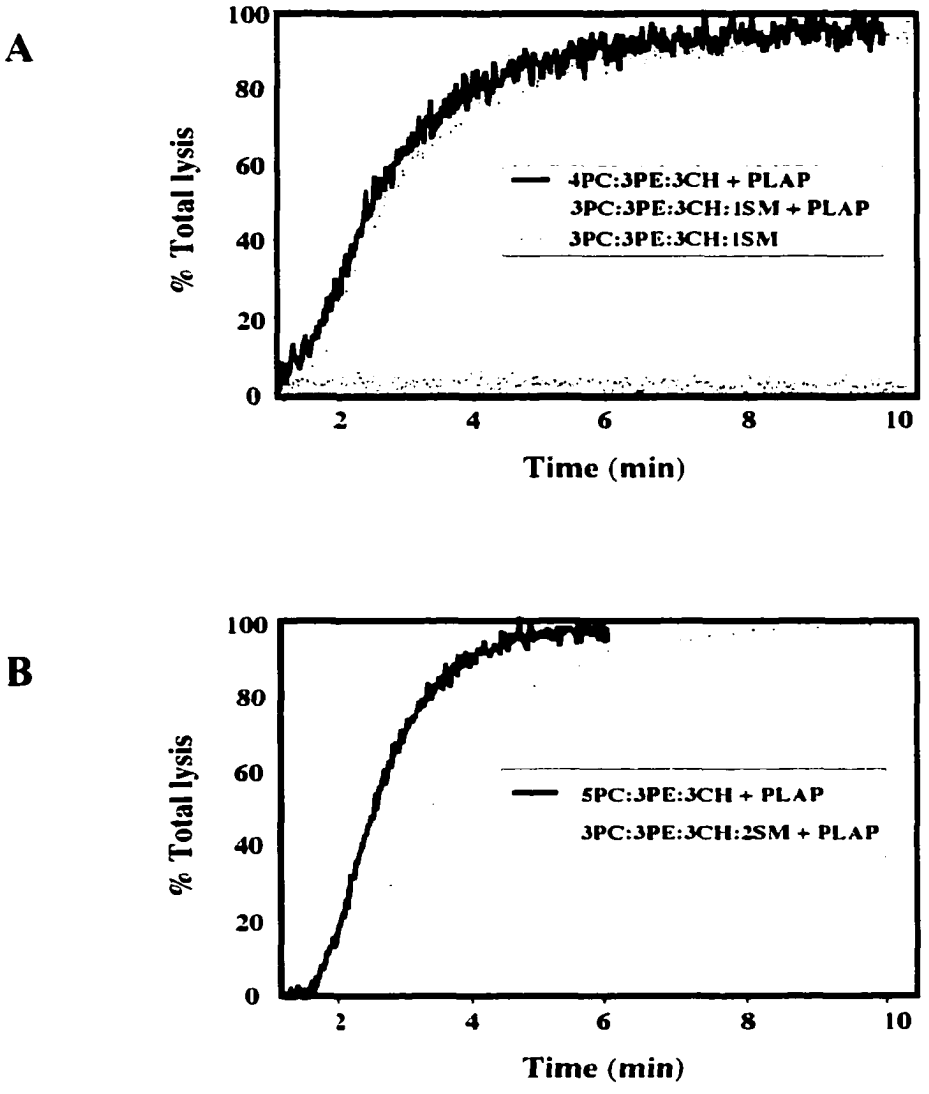


Figure 39. **Liposomes containing PLAP in rafts are not more sensitive to aerolysin than liposomes lacking rafts**. A, the rates of aerolysin-induced dye release from 3PC:3PE:3CH:1SM liposomes containing moderate raft-associated PLAP and 4PC:3PE:3CH without raft domains (overlapping curves). The flat dotted curve at the bottom was obtained with PLAP-free 3PC:3PE:3CH:1SM liposomes. *B*, sensitivity of liposomes containing PLAP that is nearly totally in raft domains (3PC:3PE:3CH:2SM) as compared to liposomes lacking raft domains (5PC:3PE:3CH) to aerolysin. Liposomes containing sphingomyelin were 145 nm in diameter, while those lacking sphingomyelin were 195 nm in diameter. Both liposomes preparations contained 1 receptor/ 1850 nm^2 of liposome surface area. Liposomes were treated with 36 nM aerolysin as described in the methods. Similar results were obtained at 37 °C.

Once PLAP had been incorporated into liposomes containing sphingomyelin, it was necessary to check that it could be recovered in detergent insoluble domains, which would indicate that it was associated with rafts. Two different liposome populations containing rafts were generated using different amounts of sphinogomyelin. Most of the PLAP in the liposomes containing phosphatidylcholine (PC), phosphatidylethanolamie (PE), cholesterol (CH), and sphingomyelin (SM), in a ratio of 3PC:3PE:3CH:2SM, was detergent insoluble, evidence for the presence of rafts, whereas in liposomes lacking SM (and therefore lacking rafts), PLAP was completely soluble in Triton X-100 (Figure 40). The amount of SM in liposomes had an effect on PLAP solubility, as liposomes containing half as much SM (3PC:3PE:3CH:1SM), had less detergent insoluble PLAP (data not shown).

The action of aerolysin on the different liposomes was next compared. The results in Figure 39 demonstrate that there were only minor differences in the rate of dye release from liposomes, regardless of their lipid composition and raft content. In fact, liposomes in which PLAP was almost entirely associated with detergent insoluble domains were less sensitive to aerolysin than those lacking raft domains (Figure 39B). These results indicate that aerolysin activity is not promoted by raft domains.

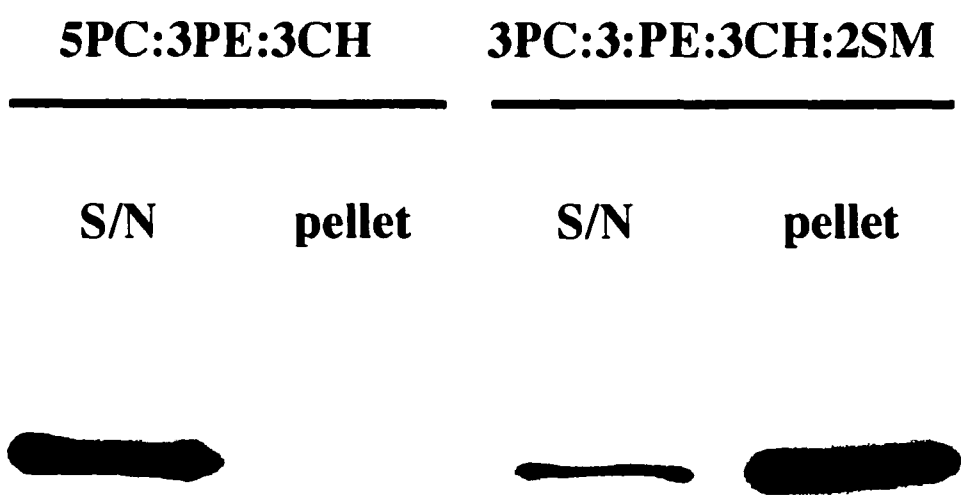


Figure 40. **PLAP can be incorporated into rafts in liposomes**. Solubility of PLAP in liposomes with (3PC:3PE:3CH:2SM) and without (5PC:3PE:3CH) detergent insoluble domains. Liposomes were solublized in Triton X-100 and detergent insoluble material was pelleted as described in the text.

DISCUSSION

The results described in this thesis provide further insight into the mode of action of aerolysin. In particular, the importance of GPI-APs in the binding of the toxin has been well demonstrated. Binding to these proteins concentrates the toxin on the cell surface facilitating oligomerization. Following oligomerization, the heptamer inserts into the cell membrane forming a functional channel. At high toxin concentrations this results in cell death by necrosis, however at lower concentrations, perhaps closer to those that might be expected at the site of an infection, the results presented here show that cell death proceeds by apoptosis. It has been suggested that the association of the toxin with GPI-APs in raft domains also promotes channel formation (Abrami and van der Goot, 1999), however my results clearly demonstrate that this is not the case.

Glycosylphosphatidylinositol-anchored proteins are receptors for aerolysin

The first aerolysin receptor that was identified was a 47-kDa glycoprotein on rat erythrocytes that has been called EAR (Cowell *et al.*, 1997; Gruber *et al.*, 1994). I wished to determine if erythrocytes from other species expressed a related receptor. Sandwich Western blots of bovine and human erythrocyte membranes revealed that they contained a proaerolysin-binding protein migrating slightly higher than EAR, whose molecular mass deglycosylated matched that of the rat erythrocyte receptor (Figure 20). Phosphatidylinositol-specific phospholipase C treatment revealed that the bovine protein was also GPI-

anchored. Although the human protein was not released by PI-PLC this was not taken as evidence that it was not GPI-anchored; two other GPI-APs that have been characterized on human erythrocytes contain an acylated inositol that makes them resistant to release by PI-PLC (Deeg *et al.*, 1992). This modification may be a characteristic of human erythrocyte proteins. The above results led me to conclude that human and bovine erythrocytes contain a homologue of the rat receptor. Expansion of the search to include nucleated cells resulted in the identification of Thy-1 on T lymphomas. Thy-1 is the most abundant GPI-AP known to be present on the surface of these cells (10^6 copies/cell) and it was by far the major proaerolysin-binding protein in sandwich Western blots of whole cell lysates (Figure 5). The search for aerolysin receptors was then extended to include other cell types as well as purified GPI-APs. We found that proaerolysin bound to contactin on brain from a variety of species, to GP63 and Thy-1 expressed on CHO cells, and to cathepsin D expressed on COS cells (Figures 8, 18, and 23; Diep *et al.*, 1998a; Nelson *et al.*, 1997). The toxin also bound to purified human placental alkaline phosphatase (PLAP), and to VSG from trypanosomes (Figure 39; Diep *et al.*, 1998a).

Glycosylphosphatidylinositol anchored proteins are excellent targets for a toxin like aerolysin, which must bind to the cell surface and oligomerize. They are found on the exterior surface of the plasma membranes of most if not all eukaryotic cells (McConville and Ferguson, 1993). In addition, their preferential localization to the apical surface of polarized epithelial cells makes them accessible to toxins secreted in the intestinal tract (Lipardi *et al.*, 2000), and the

numerous GPI-APs that have been identified on hematopoietic cells (Boccuni *et al.*, 2000) provide a variety of targets for toxins released into the bloodstream by invading bacteria.

Two different techniques were developed to demonstrate that GPI-APs not only could bind aerolysin but actually could function as receptors on a membrane surface. They involved incorporating purified GPI-APs into bilayers or membranes and then monitoring their sensitivity to aerolysin. In the first method, rat EAR was incorporated into planar lipid bilayers. The presence of this receptor lowered the concentration of aerolysin required for channel formation by more than 10^2-fold (Cowell *et al.*, 1997; Gruber *et al.*, 1994). This observation was remarkable for two reasons. First, it demonstrated that this GPI-AP was indeed a receptor for aerolysin. Secondly and equally impressively, this was the first example of a mammalian protein receptor for a bacterial channel-forming toxin being incorporated into and functioning in lipid bilayers (Gruber *et al.*, 1994). The principle behind this technique was used to develop a simpler liposome based assay (Nelson *et al.*, 1997; Figure 39), which did not require the cumbersome setup associated with planar lipid bilayers. I found that liposomes containing GPI-anchored Thy-1 or PLAP in their membranes were also at least 10^2-fold more sensitive to aerolysin than liposomes lacking receptors (unpublished). I then showed that the liposome assay can be used to study the properties of bilayers with different lipid compositions, corresponding to different domains in the plasma membrane (Figure 39). This has proven to be a valuable tool to investigate receptor-mediated aerolysin activity on model membranes.

Having demonstrated that GPI-APs could function as receptors for aerolysin on model membranes, I wished to determine if these proteins also promoted aerolysin activity on cells. The results indicated that this is the case; AKR1 T lymphomas treated with PI-PLC were 10^2-fold less sensitive to aerolysin than untreated cells (Figure 12). As mentioned previously, PI-PLC treatment is never 100 % efficient; therefore, to get a more accurate indication of the importance of GPI-APs in aerolysin activity, T lymphomas with and without GPI-APs were treated with aerolysin. This revealed that cells expressing GPI-APs (EL4) were 10^4-fold more sensitive to aerolysin than mutant cells lacking these proteins (EL4 (Thy-1-f); Figure 14). Since aerolysin activity involves binding, oligomerization and insertion, the increased sensitivity of cells expressing GPI-APs could be the result of them promoting any one of these steps.

Glycosylphosphatidylinositol-anchored proteins concentrate aerolysin on the cell surface

The most obvious way for GPI-APs to promote aerolysin activity is by facilitating binding of aerolysin to the cell surface. This was illustrated by the fact that T lymphomas expressing GPI-APs bound more proaerolysin than corresponding cells lacking them, as determined by confocal microscopy and flow cytometry (Figures 15 and 16). Since binding to GPI-APs concentrates aerolysin on the cell surface, and oligomerization is a concentration dependent process, it is likely that binding to these proteins promotes oligomerization. This was previously demonstrated on BHK cells, where oligomerization was observed at

10^5 to 10^6-fold lower aerolysin concentrations on the cell surface than in solution (Abrami and van der Goot, 1999). The ability of GPI-APs to concentrate aerolysin very likely contributes to the 10^4-fold difference in sensitivity between cells with and without GPI-APs (Figures 14). This is presumably also the reason that erythrocytes and T lymphocytes from patients with PNH, which do not express GPI-APs, are far less sensitive to aerolysin (Brodsky *et al.*, 1999 and 2000).

Having shown that GPI-APs promoted aerolysin activity on cells, and that Thy-1 functioned as a receptor for aerolysin on liposomes, I wanted to determine the contribution of Thy-1 to the toxin's activity on cells. This was accomplished by comparing the sensitivity of cells with and without Thy-1 to aerolysin (AKR1 and AKR1(Thy-1-d); Figure 11). Surprisingly, I found that cells lacking Thy-1 were as sensitive to the toxin as normal cells (Figure 11). As well, I could detect no difference in the binding of FLAER to these cells by confocal microscopy or flow cytometry (data not shown). Treatment of AKR1 (Thy-1-d) cells with PI-PLC revealed that their sensitivity to aerolysin was due to the presence of other GPI-anchored aerolysin receptor(s) (Figure 11). A clue to the identity of these receptors was provided by a sandwich Western blot of concentrated membrane proteins, which revealed proaerolysin-binding proteins migrating at 30 and 70-kDa (Figure 12). There are at least ten different GPI-APs on T lymphocytes (Boccuni *et al.*, 2000), some of which may be the proteins observed in Figure 12. They include CAMPATH-1, which is present at 5 x 10^5 copies/cell and migrates at 26-kDa, and CD55 and CD73, which both migrate at 70-kDa (Madrid-Marina *et al.*, 1993). It is not surprising that at least one and possibly several GPI-APs are

able to function as receptors in the absence of Thy-1. The sum total of alternate GPI-anchored receptors, which may include as yet unidentified GPI-APs may be comparable to the amount of Thy-1 on the cell surface. These alternate receptor(s) may be able to concentrate a sufficient amount of aerolysin during the 1 hour incubation with toxin to allow for a comparable amount of oligomerization and subsequent cell death. Alternatively, the assay used here may not be able to measure a difference in sensitivity between these cells.

Glycosylphosphatidylinositol-anchored proteins promote oligomerization

In addition to concentrating aerolysin on the cell surface, GPI-APs may facilitate oligomerization by allowing the toxin to move laterally. In fact, some GPI-APs, including Thy-1, have been reported to have a faster diffusion coefficient than transmembrane proteins; for others the rates are comparable (Jacobson *et al.*, 1997; Kooyman *et al.*, 1995; Simson *et al.*, 1998; Thomas *et al.*, 1990; Zhang *et al.*, 1991). To determine if toxin bound to GPI-APs oligomerized better then toxin bound to other cell surface components, oligomerization was compared at a very high toxin concentration (5×10^{-7} M) where there was a comparable amount of aerolysin bound to cells with and without GPI-APs (Figure 17). Under these conditions, significantly more oligomer was formed on cells with GPI-APs than on cells lacking them (Figure 17). Although it is tempting to speculate that the lateral mobility of GPI-APs promotes oligomerization, it is also possible that binding to GPI-APs promotes oligomerization in some other way. Further investigation will be required to determine if this is the case.

Glycosylphosphatidylinositol-anchors are binding determinants for proaerolysin

The results presented in this thesis reveal several remarkable things about the interaction of aerolysin with GPI-APs. The fact that GPI-APs could easily be detected following PI-PLC treatment indicates that the diacylglycerol is not required for binding (Figures 6 and 20). Similarly, the acyl chain bound to the inositol of some GPI-APs is not required, as the toxin bound to proteins with and without acylated inositol (Figures 22 and 24). These results are not surprising since the acyl chains are embedded in the membrane and therefore presumably inaccessible for binding. My results also show that N-linked sugars are not required for binding, as the toxin bound equally well to glycosylated and deglycosylated receptors (Figures 6, 18 and 21). Since the glycosyl portion of the GPI-anchor was the only common determinant remaining among these proteins, I concluded that it is the binding determinant for proaerolysin. This conclusion was supported by the observation that proaerolysin binding decreased following HF treatment of VSG, which removes the anchor, and by the failure of proaerolysin to bind Thy-1 expressed in *E. coli* (Diep *et al.*, 1998a).

How proaerolysin penetrates the cell surface glycocalyx to reach the anchor is not known. It is possible that the initial interaction of the toxin with the cell surface involves carbohydrate side chains on the glycocalyx. This is supported by the fact that proaerolysin is able to bind to the highly glycosylated transmembrane protein glycophorin (Howard and Buckley, 1982; Diep *et al.*,

1999). An indication of the portion of proaerolysin involved in binding to glycophorin was obtained by comparing proaerolysin, alpha-toxin binding to this protein. Although both proaerolysin and alpha-toxin bind to GPI-APs, only proaerolysin was able to bind to glycophorin suggesting that the small lobe may be facilitating binding. The small lobe likely interacts with carbohydrate determinants on glycophorin since it contains an APT domain that is suggested to be involved in carbohydrate binding (Rossjohn *et al.*, 1997a). This was confirmed by the observation that the addition of the small lobe to alpha-toxin enabled the subsequent hybrid alpha-toxin to bind to glycophorin. Surface plasmon resonance and ELISA measurements revealed that the interaction between glycophorin and proaerolysin or hybrid alpha-toxin is considerably weaker than that with GPI-APs (MacKenzie *et al.*, 1999). Since aerolysin is a dimer, binding of one monomer to a carbohydrate determinant on glycophorin or another component of the glycocalyx could form an initial interaction with the cell surface positioning the other monomer for interaction with the anchor. Binding of aerolysin to the anchor, which is laterally mobile and is located within 2 nm of the plasma membrane (Barboni *et al.*, 1995), allows oligomerization to occur close to the membrane, which should facilitate insertion of the oligomer.

It is interesting to note that aerolysin is not the only pore-forming toxin that uses GPI-anchored receptors. Alpha-toxin from *C. septicum*, which also must oligomerize to become active, uses a variety of GPI-anchored receptors, and Cry1Ac from *Bacillus thuringieneses* uses a GPI-anchored aminopeptidase in the insect gut as a receptor (deMaagd *et al.*, 1999; Gordon *et al.*, 1999). Although

there is no evidence to suggest that Cry1Ac binds to the anchor, preliminary results with aerolysin and the homologous alpha-toxin suggest that they may share a common binding domain that interacts with the anchor, as will be discussed below.

Anchor structures recognized by proaerolysin

Although GPI-APs from all species share a common core anchor, the core may be variably modified in different species and cell types by the addition of sugars or phosphoethanolamine, or by the acylation of inositol (Brewis *et al.*, 1995). The results presented here indicate that glycan core modification can have a profound effect on binding. Thus the ability of proaerolysin to bind to GP63 expressed in CHO cells, which contains a mammalian anchor, but not GP63 from *L. major* which contain a parasite anchor (Figure 24), suggested that a fundamental difference between the two anchors influences binding. The most obvious difference is that the mammalian anchor contains at least one additional phosphoethanolamine attached to the basic core anchor at carbon two of the third mannose (Figure 24), whereas the core of the parasite anchor is unmodified. This suggests that the core anchor plus phosphoethanolamine might be the minimum binding determinant that is recognized by proaerolysin.

Further evidence regarding the features of the anchor recognized by proaerolysin was obtained from a comparison of proaerolysin binding to a number of different GPI-APs. Proaerolysin bound to six of eight mammalian proteins from eight different species (Figures 10, 18, 20, 21, 23, 38; Diep *et al.*, 1998a;

see above). On the other hand, only one of three parasite proteins was recognized. The ability of proaerolysin to detect a wide range of mammalian GPI-APs but only one parasite GPI-AP provides further evidence for a difference in anchor structure between the two groups of proteins that influences proaerolysin binding. Since all mammalian proteins contain the basic core structure plus a conserved phosphoethanolamine attached to carbon two of the third mannose, this may indeed be the minimum binding determinant for proaerolysin. The one non-mammalian protein recognized by proaerolysin is VSG, which does not contain the additional ethanolamine, but instead may contain one to four sugar residues attached to the same mannose (Figure 24; McConville and Ferguson, 1993). This suggests that the mannose residue may only need to be derivatized at the carbon two or three of the third mannose, not specifically modified with phosphoethanolamine . The expression of a given proaerolysin-binding protein in different cell types, where it will receive an anchor characteristic of that cell type, should help to elucidate the anchor structures that are recognized by proaerolysin.

A suitable anchor is not sufficient for proaerolysin binding

Since anchor structure appears to be cell specific, a comparison of proaerolysin binding to GPI-APs on a given cell type can be used to study the role of the polypeptide in binding. My evidence suggests that some proteins that should have suitable anchors are not bound by proaerolysin. For example, brain NCAM is not bound by proaerolysin, whereas purified brain contactin is (Figures 18 and 19), and proaerolysin does not recognize the folate receptor expressed on

CHO cells, but does bind to GP63 and Thy-1 on the same cells (Figure 23; Diep *et al.*, 1998; Gordon *et al.*, 1999). It may be that the polypeptide structure of NCAM and the folate receptor are not recognized by proaerolysin, or that when these proteins are denatured following SDS PAGE they are unable to refold into their native structure for recognition. Alternatively, some portion of these proteins may hinder access of the toxin to the anchor. Whatever the case, these results demonstrate that the polypeptide chain is in some way involved in proaerolysin binding.

Additional evidence supporting a role for the protein in binding was provided by the observation that binding to the rat erythrocyte receptor is reduced by protease treatment (Cowell *et al.*, 1997). Thus trypsin treatment of erythrocyte membranes incorporated into planar lipid bilayers, resulted in decreased sensitivity of these bilayers to aerolysin (Cowell *et al.*, 1997). We also observed that protease treatment of liposomes containing Thy-1 reduced their sensitivity to aerolysin (Diep *et al.*, 1999). The generation of hybrid GPI-APs with different polypeptide chains may help to determine what protein structures are required for proaerolysin recognition. Preliminary experiments have been preformed in which a GPI-anchor signal sequence was fused to the soluble protein cathepsin D and expressed in COS cells. The resulting hybrid protein was bound by proaerolysin whereas the soluble form was not. This demonstrates that whatever the contribution of the polypeptide is to binding it is not specific to GPI-APs and that this technique can be used to study the requirement for proaerolysin binding.

The protein structures of some of the GPI-APs that are bound by proaerolysin have been solved; however they provide no obvious answer as to their contribution to proaerolysin binding. A comparison revealed that three of them share some structural similarity. Contactin, Thy-1 and VSG all contain an immunoglobulin-like domain fold, which consists of a sandwich of two antiparallel β-sheets (Leahy, 1997); however NCAM also contains this fold and it is not recognized by proaerolysin, at least after SDS PAGE. Furthermore, PLAP and GP63, which are bound by proaerolysin, do not contain this fold. Further work is required to determine what role protein structure plays in binding.

Domains on proaerolysin involved in receptor binding

The ability of proaerolysin to recognize the GPI-anchor, a portion of the polypeptide chain of GPI-APs, and at least one transmembrane glycoprotein suggests that more than one domain on the toxin is involved in interacting with receptors. This is also supported by the evidence that both the large and small lobe of aerolysin appear to be involved in binding.

Evidence involving the large lobe in binding to GPI-APs has come from a variety of sources. First, alpha-toxin from *C. septicum*, which shares extensive sequence homology with the large lobe of aerolysin, also binds GPI-APs (Gordon *et al.*, 1999). Second, replacing aromatic residues on the top face of the large lobe (Y162A and W324A) results in decreased binding to GPI-APs (MacKenzie *et al.*, 1999). Finally, the large lobe expressed without the small lobe was able to bind to

GPI-APs, although the binding affinity was 50-fold lower than for native aerolysin (Diep *et al.*, 1999; MacKenzie *et al.*, 1999).

The small lobe has a similar fold to the C-type lectins, which are known to bind carbohydrate, suggesting that it too may be involved in carbohydrate binding (Rossjohn *et al.*, 1997a). Based on close structural similarity with the binding domain of pertussis toxin this fold has been called an APT domain (Rossjohn *et al.*, 1997a). The mutation of any of five conserved surface exposed residues in the APT domain (W45A, I47E, M57A, Y61A, and K66Q) results in a 25 to 250-fold decrease in proaerolysin binding to Thy-1 (MacKenzie *et al.*, 1999). Also, as mentioned above a hybrid toxin in which the small lobe of aerolysin was fused with alpha-toxin had increased binding affinity for GPI-APs and glycophorin (Diep *et al.*, 1999). Although these results demonstrate that the small lobe is also involved in binding, the small lobe expressed by itself binds to GPI-APs with a 5000-fold lower affinity than native aerolysin.

We found that a double mutant in which critical residues were mutated in both the large and small lobe (Y61A/W324A) was unable to bind to GPI-anchored receptors and was correspondingly inactive against T lymphomas (MacKenzie *et al.*, 1999; unpublished). Also, as mentioned above hybrid alpha-toxin had an increased binding affinity for GPI-APs and glycophorin (Diep *et al.*, 1999). These results demonstrate that regions on both the large and small lobe work together to facilitate binding. It is yet to be determined whether these two regions come together to form one binding domain or whether each domain contacts a separate binding determinant.

Raft Domains do not Promote Aerolysin Activity

The structure of raft domains is not conducive to aerolysin activity

The disruption of raft domains on T lymphomas by cholesterol extraction with methyl-β-cyclodextrin had no effect on the sensitivity of the cells to aerolysin (Figure 38), nor did similar treatment of erythrocytes decrease the rate of cell lysis by the toxin. Similarly, liposomes with and without raft domains were equally sensitive to aerolysin. Therefore, in contrast to the published suggestion of Abrami and van der Goot, I concluded that the association of aerolysin with these domains does not enhance the rate of channel formation (1999; Figure 39). In fact, since receptor binding appears to be the rate-limiting step in aerolysin activity at low toxin concentrations (Garland and Buckley, 1988), it is unlikely that the association of the toxin with raft domains would have any measurable effect on the kinetics of oligomerization. It could be argued that the tight packing of acyl chains in raft domains could reduce channel formation under some conditions. First, the lateral mobility of GPI-APs may be reduced due to the tight packing of saturated sphingolipids and cholesterol in these domains (Sheets *et al.*, 1997; Simson *et al.*, 1998; Swamy *et al.*, 1999). This reduced mobility may inhibit oligomerization by reducing the rate at which molecules of aerolysin would encounter one another. Second, the raft acyl chains might inhibit insertion of the oligomer into the membrane, since insertion into a tightly packed liquid ordered domain might be more difficult than insertion into a relatively fluid liquid crystalline domain. These properties may contribute to the decreased sensitivity of

liposomes containing a high proportion of rafts to aerolysin observed in Figure 39B.

Cholesterol is not required for aerolysin activity

A recent report that aerolysin activity is inhibited following incubation with free cholesterol led to the proposal that the toxin may interact with this steroid (Ferguson *et al.*, 1997). This proposal is not supported by the observation that aerolysin forms channels in cholesterol free liposomes (Howard and Buckley, 1982) and my evidence that cholesterol depleted cells are not less sensitive to aerolysin (Figures 36 and 38). In fact, I found that the removal of nearly all of the cholesterol from erythrocytes did not decrease the rate of channel formation by aerolysin (Figure 38). Thus it would appear that cholesterol plays no significant role in aerolysin activity.

Aerolysin induces Apoptosis

The ability of aerolysin to lyse erythrocytes has long been recognized (Bernheimer *et al.*, 1975). Several studies including those reported in this thesis have been made with other cells including T lymphomas that demonstrate that aerolysin activity is not restricted to erythrocytes, nor is the mechanism of cell death restricted to necrosis (Figures 12 and 14; Abrami *et al.*, 1998; Gordon *et al.*, 1999;). Although treating T lymphomas with high concentrations of aerolysin results in cell death by necrosis, I showed that at low concentrations (10^{-10} M) cell death proceeded by apoptosis (Figure 25A and B). Results with aerolysin variants

deficient in various stages of oligomerization or insertion demonstrated that apoptosis was not triggered by signaling induced by toxin binding or toxin-induced clustering of GPI-APs (Figure 29). Additionally, the observation that aerolysin could induce apoptosis in cells lacking GPI-APs (Figure 30) eliminated the possibility that signaling mediated specifically by GPI-APs was triggering apoptosis.

Channel formation is required for aerolysin-induced apoptosis

Since I could show that GPI-APs were not directly involved in aerolysin-induced apoptosis, it seemed reasonable to conclude that channel formation was required as previously demonstrated for other pore-forming toxins (Chen and Zychlinsky, 1994). One consequence of channel formation is an increase in intracellular calcium, as the cation enters the cell from the external media. An intracellular calcium indicator was used to demonstrate that the concentration of this cation increased at the toxin concentration where apoptosis was observed (Figure 31). Since both the influx of calcium from the external media through aerolysin channels and the release of calcium from the ER that occurs during apoptosis could result in elevated calcium, it was necessary to determine its source. Results obtained with cells incubated in calcium free media demonstrated that the increase was a result of the cation entering the cells from the external media through channels in the membrane and not a result of the mobilization of intracellular calcium stores (Figure 32). This result is not consistent with the claim of Krause *et al.* that the increase in intracellular calcium in granulocytes

following aerolysin treatment is the result of the toxin triggering the release of calcium from intracellular stores (1998). In addition, these studies demonstrated that the time required to permit the maximum increase in intracellular calcium increased with decreasing aerolysin concentration (data not shown). This is consistent with the expectation that a smaller number of channels are formed at lower toxin concentrations.

It seems likely that aerolysin only induces apoptosis at low concentrations because at toxin concentrations above 10^{-10} M so many channels are formed that the cells die very quickly and the apoptotic pathway is either not triggered, or there is no time for its effects to be observed. Thus at low toxin concentrations (10^{-10} M) enough channels are formed to trigger apoptosis by causing a change in the concentration of cations such as potassium or calcium within the cell, but not so many that the cell cannot survive to initiate an apoptotic response (Bortner *et al.*, 1997; Squier *et al.*, 1994 and 1999). The number of channels formed at the concentration where apoptosis is observed is likely similar on cells with and without GPI-APs, since in both cases this is the lowest concentration required to cause 100 % cell death. To investigate this likelihood, the amount of time required to cause a maximum increase in intracellular calcium at the toxin concentrations where apoptosis was observed could be compared. If the maximum calcium influx occurred at the same time for both cell types, this would provide further evidence for the mechanism of aerolysin-induced apoptosis.

The induction of apoptosis by other pore-forming bacterial proteins, including *S. aureus* alpha-toxin, *E. coli* HlyA, *Actinobacillus*

actinomycetemcomitans leukotoxin, and *Neisseria gonorrhoeae* porin Por B, also occurs at low toxin concentrations (reviewed in Chen and Zychlinsky, 1994; Muller *et al.*, 1999). It has been suggested that the elevation of intracellular calcium levels associated with channel formation may trigger apoptosis by the activation of calcium dependent proteases such as calpain which are involved in apoptotic signaling (Muller *et al.*, 1999; Squier *et al.*, 1994 and 1999). Further work is required to determine if calpain or any other calcium dependent enzymes are involved in aerolysin-induced apoptosis. Aerolysin will be another useful tool in the study of how a limited amount of membrane permeabilization is able to initiate an apoptotic response.

Benefits of aerolysin-induced apoptosis

Apoptotic cell death of circulatory cells offers several advantages to the invading bacteria. It reduces the likelihood of necrosis and avoids a localized inflammatory response resulting from rupture of the host cells. In addition, the destruction of immune system cells reduces the chance that they will be available to attack the invading bacteria at the site of an infection. It is tempting to speculate that apoptosis might more likely be the mechanism of cell death induced by aerolysin *in vivo*, since the concentration of aerolysin at the site of an infection is presumably quite low.

Potential Applications

Due to its high affinity binding to GPI-APs (K_D 10^{-8} M), aerolysin makes a useful tool for their study. The interaction has already been exploited for a variety of purposes including the detection of GPI-APs, and the diagnosis of PNH.

Glycosylphosphatidylinositol-anchored protein detection

Prior to the studies described here, there were three rather unsatisfactory methods available for the detection of GPI-APs. The most direct method employed an antibody (anti-CDR) raised against PI-PLC treated VSG. Two other more indirect methods employed surface biotinylation, followed by PI-PLC treatment and detection of released proteins by enzyme-linked streptavidin, or metabolic labeling of GPI-APs with radiolabeled ethanolamine or inositol. The anti-CRD antibody has two serious limitations. First, the limit of detection is quite high, typically 0.2 μg of protein or more is required (Figure 22), which often requires that the protein must be concentrated. Second, the protein must be PI-PLC treated before detection as the antibody will not react with an intact anchor. Although surface biotinylation and metabolic labeling offer a high degree of sensitivity, they are both relatively labour intensive methods. The use of proaerolysin to detect GPI-APs offers several advantages over the low affinity antibody and the indirect labeling methods. First, very low levels of purified GPI-APs or proteins in whole cell lysates can be detected with aerolysin (Figures 5 and 10). Second, PI-PLC treatment is not required for detection, as the protein with an intact anchor is detected just as well as the enzyme treated form (Figures 6, 18

and 21). Disadvantages to using aerolysin are that not all GPI-APs are bound by the toxin. However, this method provides another useful tool for the detection of some GPI-APs.

Ligand for GPI-mediated signaling

Binding and crosslinking of GPI-APs, especially on T lymphomas, has been shown to trigger intracellular signaling (reviewed in Brown, 1993). Since ligands for most GPI-APs have yet to be identified, monoclonal or polyclonal antibodies are often used as substitutes, with crosslinking sometimes brought about by a secondary antibody (Brown, 1993; Horejsi *et al.*, 1998). In contrast, aerolysin provides a one-step mechanism to crosslink GPI-APs on the cell surface. Although aerolysin does not share the specificity of antibodies for individual GPI-APs. it can provide general information about GPI-mediated signaling. In addition, aerolysin variants that are deficient in oligomerization or insertion into the membrane do not cause cell lysis and will be useful tools in determining if an intracellular signal is the result of binding or crosslinking of GPI-APs.

Inactivation of HIV infectivity

Another unique property of aerolysin is its ability to inactivate HIV viral particles. During its release from infected cells the virus incorporates host cell membrane containing GPI-APs into its envelope; these include the aerolysin receptor Thy-1 and CD59 (Nguyen *et al.*, 1999). Previously, the binding of

aerolysin to Thy-1 on liposomes was shown to promote oligomerization and channel formation (Nelson *et al.*, 1997). Oligomerization also occurs on the surface of viral particles destroying the infectivity of HIV. How the insertion of the 350-kDa heptamer into viral membranes blocks the infectivity of these particles is yet to be elucidated (Nguyen *et al.*, 1999). It has been suggested that the insertion of the oligomer into the viral membrane may interfere with the viruses' ability to fuse with target cell membranes (Nguyen *et al.*, 1999); however this requires further investigation.

Diagnosis and study of PNH

Differential binding and sensitivity to aerolysin has been exploited to detect populations of cells not expressing GPI-APs in blood from patients with PNH (Brodsky *et al.*, 1999; Brodsky *et al.*, 2000). Paroxysmal nocturnal hemoglobinuria is a disease characterized by a defect in the expression of GPI-APs on hematopoietic cells. Aerolysin can be used to detect these cells using a spectrophotometric assay to monitor aerolysin induced lysis of erythrocytes or by measuring the binding of FLAER to lymphocytes and granulocytes by flow cytometry (Brodsky *et al.*, 1999; Brodsky *et al.*, 2000). In both cases a comparison between PNH and normal blood from a healthy donor is made. Populations of affected cells as small as 0.1 % can be detected in this manner (Brodsky *et al.*, 1999; Brodsky *et al.*, 2000).

The dramatically lower sensitivity of PNH cells to aerolysin raises an interesting possibility regarding the development of PNH. It is known that the

disorder arises from one or more hematopoietic stem cells containing a mutated *PIG-A* gene (reviewed in Rosti, 2000). Although the genetic defect and phenotype of PNH cells have been well characterized, little is known about how these abnormal cells gain an advantage over normal cells (Bocuni *et al.*, 2000). Several hypotheses have been put forward. First, it has been suggested that the deficiency in GPI-APs gives the PNH clone a growth advantage. However, this theory is not supported by the fact that the PNH clone coexists for a long time with normal cells (Rosti, 2000) and PNH cells have been detected at very low numbers in normal individuals (Araten *et al.*, 1999). This suggests that some change is required that favours expansion of the PNH clone. Support for this theory comes form the observation that PNH often gives rise to aplastic anemia or *vice versa* (Schubert *et al.*, 1994; Socie *et al.*, 1996). Aplastic anemia (AA) is a hematological disorder characterized by a deficiency in the production of erythrocytes, lymphocytes and platelets (Nissen, 1991). The theory linking PNH and AA proposes that the expansion of the PNH clone is favoured by an additional environmental change in the body, which may include an injury to normal hematopoiesis that does not affect the PNH clone (Griscelli-Bennaceur *et al.*, 1995). This theory is supported by the fact that aplastic anemia often arises following bone marrow damage (Rotoli *et al.*, 1993). An alternative theory regarding the expansion of the PNH clone is related to the work described in this thesis. Some cases of PNH could arise following septicemia caused by *A. hydrophila*, *C. septicum*, or other bacteria that produce toxins that bind GPI-anchored proteins. These toxins bind specifically to GPI-APs on hematopoietic

cells among others and oligomerize to form channels, which can result in the death of normal erythrocytes and lymphocytes. Also, since stem cells contain GPI-APs, including low levels of Thy-1, the toxins may also kill them. These toxins would also provide a positive selection pressure for PNH cells as they would be unaffected. To test this theory hematopoietic cells could be grown in the presence of either toxin. It was previously shown that mutated CHO cells could be cultured in the presence of alpha-toxin to select for clones that were resistant to the toxin (Gordon *et al.*, 1999). Like PNH cells these clones lacked the ability to synthesize GPI-anchors (Gordon *et al.*, 1999). It will be interesting to determine if this method can also be used to select for rare PNH clones in populations of hematopoietic cells.

Summary

In summary, the results presented in this thesis give new insight into the mode of action of aerolysin. In particular, the importance of GPI-APs in promoting aerolysin activity has been clearly demonstrated. The organization of these proteins in raft domains on the cell surface appears to have no affect on aerolysin activity; their mere ability to bind to aerolysin seems to promote oligomerization. Finally, it has been demonstrated that aerolysin not only kills cells by cytolysis, but that it also can induce apoptosis at low concentrations. Although GPI-APs have also been shown to be involved in inducing apoptosis, here they seem to only play a role in promoting channel formation.

Future Direction

In addition to clarifying the stages in the mode of action of aerolysin, the work presented here has raised several new questions including: How does aerolysin interact with GPI-anchors? Can purified GPI-anchor alone function as a receptor for aerolysin? What contribution does the polypeptide portion of the receptor make to binding? How does binding to GPI-APs promote oligomer formation? How does the formation of a small number of channels induce apoptosis? These questions suggest new avenues for future research on the mode of action of aerolysin.

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