Identification and Characterization of the Aerolysin Receptor from Rat Erythrocyte Membranes

by

Simon Piers Cowell
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Dr. J.T. Buckley, Supervisor (Department of Biochemistry and Microbiology)

Dr. R.W. Olafson, Departmental Member (Department of Biochemistry and Microbiology)

Dr. D.B. Levin, Outside Member (Department of Biology)

Dr. S. Misra, Additional Member (Department of Biochemistry and Microbiology)

Dr. M.E. Corcoran , External Examiner (Department of Psychology)

University of Victoria

Supervisor: Dr. J. T. Buckley

ABSTRACT

A protocol was devised for the isolation of a 47 kDa glycoprotein from the rat erythrocyte membrane which was able to bind the Gram negative bacterial protein toxin aerolysin with high affinity. The protein was extracted in non-ionic detergent and purified using ion-exchange chromatography. This was followed by affinity chromatography using immobilized wheat germ agglutinin. A partial N-terminal sequence was obtained for the purified protein. It revealed homology with a family of glycosylphosphatidylinositol-anchored proteins including muscle cell ADP-ribosyltransferases and rat and mouse T-cell antigens. The 47 kDa aerolysin binding protein (ABP) was released into the supernatant during treatment of rat erythrocyte membranes with phosphatidylinositol specific phospholipase C (PI-PLC; which cleaves GPI-anchors). This further supported the classification of the rat erythrocyte ABP as a novel member of this protein family. The treated rat erythrocytes were less sensitive to aerolysin than control cells not treated with the enzyme. Supernatants containing the ABP were able to inhibit toxin-mediated haemolysis after preincubation with aerolysin. Analysis of a number of other cell lines revealed that rat T-cell line EL-4, and mouse erythrocytes also possess a single high-affinity aerolysin binding species.

The effects of enzymatic treatments upon the protein's aerolysin binding activity were examined. Treatment of the ABP with peptide N-glycosidase F removed N-linked oligosaccharides, and revealed that the protein carries two or more such moieties, but that these are not required for aerolysin binding. The protein was found to be sensitive to

trypsin but not to chymotrypsin, and trypsin treatment abolished aerolysin binding activity.

Examiners:

Dr. J.T.Buckley, Supervisor (Department of Biochemistry and Microbiology)

Dr. R.W.Olafson, Departmental Member (Department of Biochemistry and Microbiology)

Dr. D.B.Levin, Outside Member (Department of Biology)

Dr. S.Misra, Additional Member (Department of Biochemistry and Microbiology)

Dr. M.E. Corcoran, External Examiner (Department of Psychology)

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LIST OF ABBREVIATIONS

ABP	=	Aerolysin binding protein
ADP-RT	=	Adenosine diphosphate ribosyltransferase
CAPS	=	3-[Cyclohexylamino]-1-propanesulfonic acid
CHAPS	=	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.
DEAE	=	Diethylammonioethyl
DMF	=	Dimethyl formamide
dH_2O	=	Deionised water
E. coli	=	*Escherichia coli*
EDTA	=	Ethylenediaminetetraacetic acid
EtOH	=	Ethanol
GluNAc	=	N-acetyl glucosamine
HBA	=	Human blood agar
HEPES	=	N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]
Iodogen	=	1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril
IPTG	=	Isopropyl-D-thiogalactopyranoside
kDa	=	kiloDalton
LIS	=	Lithium di-iodosalicylate
MeOH	=	Methanol
OD	=	Optical Density
PI-PLC	=	Phosphatidylinositol-specific phospholipase C
PMSF	=	Phenylmethylsulfonyl fluoride
PNGaseF	=	Peptide-N-glycosidase F
PVDF	=	Polyvinylidene difluoride
RBCM	=	Red blood cell membranes
RT	=	Room temperature (usually 20 to 23°C)
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	SDS-polyacrylamide gel electrophoresis

SLS	=	Sodium N-laurylsarcosinate
t	=	Time of incubation
Tris	=	Tris[hydroxymethyl]aminomethane
Tween-20	=	Polyoxyethylene-20-sorbitan monolaurate
WGA	=	Wheat germ agglutinin
WT	=	Wild type

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DEDICATION

In loving memory of my grandmother, Dorothy Chamberlain, who finally found peace on the day I received my long sought after N-terminal sequence data. She always encouraged her grandchildren to take full advantage of the educational opportunities which she had been denied, and was always proud of our achievements.

INTRODUCTION

Bacterial Protein Toxins

Many bacteria, both Gram positive and negative, secrete proteins into the extracellular milieu. Among these proteins are many that are toxic to eukaryotic cells, and which are important or essential virulence factors of pathogens. The importance of bacterial diseases in humans, and in the plants and animals on which we depend for food, has ensured that these microbial virulence factors, and toxins in particular, have been a focus for research since the earliest days of science and medicine, and will continue to be so long into the future. Toxins are used by bacteria for a number of purposes, and many bacteria produce several toxins to fulfill different roles. Consequently, the cellular response to a toxin is almost as variable as the individual toxins themselves and can also depend upon the cell line affected. The stimulation of fluid secretion from the enterocyte cell seen as a result of many enterotoxins, and symptomatic of diarrhea, may help the bacteria to disperse into the environment to infect new hosts. The lysis of target cells by toxins may be a mechanism to increase the availability of nutrients for bacterial growth; alternatively, it may be a mechanism to assist bacterial invasion, or to suppress the host's immune response.

Many of the toxic protein products of bacterial cells are non-specific in their action; they remain in the extracellular environment and are not targeted to bind and act upon specific host cell types. These include many enzymes such as lipases and proteases which can have a general degradatory effect upon the integrity of host cell membranes. This thesis concerns the binding of the secreted bacterial protein toxin aerolysin to host

cell membranes via a specific interaction and the role of binding in toxicity. In this introduction, emphasis is placed upon those toxins that, like aerolysin, must bind to and penetrate the target cell membrane.

Membrane-penetrating bacterial protein toxins can be divided into two groups according to their modes of action. One group consists of toxins that must gain access to the cytosol of their target cells in order to be active. The second group comprises toxins that interact directly with the target cell membrane and do not require translocation into the cytosol.

Although there is considerable variation in the mechanisms employed by the various protein toxins, there are typically five shared, basic steps along the route leading to the disruption of the target cells: synthesis, secretion, target cell recognition, activation and membrane insertion. These steps will be described below, and selected example toxins will be described in more detail later in this introduction.

The genes encoding toxins may be located in the genome or carried on a plasmid. Sometimes a gene is found as part of an operon encoding a number of proteins that may have apparently unrelated functions or may assist in the subsequent processing or secretion of the toxin. Details of the regulation of toxin gene expression are not well defined for most toxins. The mRNA transcript is translated into a polypeptide chain which must be folded and processed to yield the secreted form of the toxin. The degree and nature of this processing varies considerably from one toxin to another, but typically includes proteolytic cleavage and the formation of disulfide bridges. Most toxins are

initially secreted as inactive precursors which require some extracellular processing to fully activate toxic activity. Often the toxin is not processed to a fully active form until bound to the target cell, presumably so that the bacterium itself is not put at risk.

The process of secretion of toxins from bacterial cells, particularly Gram negative organisms which have two membranes to traverse, has been an area of considerable research interest. Several mechanisms have been elucidated in different bacterial species designed to complement the particular needs and limitations of the bacteria. Some species have been found to utilize multiple secretory pathways for their secreted proteins (Salmond and Reeves, 1993; Pugsley, 1993).

Many toxins can recognize, and bind to, the plasma membranes of target cells. Current evidence indicates that each toxin binds to a particular species of receptor molecule or family of molecules on target cell types. The receptor may be a protein, lipid, glycoprotein, glycolipid or a complex of more than one of these. The specificity of the toxin-receptor interaction varies greatly from toxin to toxin. Some require only a simple motif for binding, which may be found in a great variety of cell surface components in many or all cell types. Such toxins will have a broad range of target cell lines. Other toxins are able to bind only one specific type of molecule that may only occur on particular cell lines or at certain developmental stages, making these toxins highly specific for certain cells. For a number of toxins, the receptor may function as more than just a means for the toxin to recognize and bind to a target cell. The mobility of receptor-toxin complexes on the plasma membrane often appears to be important (Spiegel *et al.*, 1984), suggesting that receptor binding provides an effective means of

concentrating the toxin on the cell surface and bringing together individual toxin molecules so that aggregation can occur. In addition, for some toxins the receptor may have an important role in subsequent membrane insertion steps (Fishman, 1990). Also, binding to the receptor may be required to induce conformational changes in the toxin which then initiate activation.

Once in position on the cell membrane, the toxin must become fully activated so that it can interfere with normal cellular functioning. Often this processing involves or stimulates some form of structural reorganization of the toxin: one part or subunit may be released, hydrophobic surface charge distribution may change, or oligomerization may occur. For the enzymatic toxins, the active domain of the toxin must be released into the cytosol, and this may require the reduction of one or more disulfide bonds. For the pore-forming toxins, activation may involve proteolytic cleavage, which stimulates structural changes and often oligomerisation. Oligomerization and channel formation are by no means restricted to cytolytic toxins. Many of the enzymatic toxins have been found to produce ion channels in bilayers which are thought to represent an essential part of the translocation machinery.

For all bacterial protein toxins discussed here, part of the toxin must enter the bilayer of the target cell membrane at some stage, moving from the hydrophilic extracellular milieu to the hydrophobic membrane core. For the first group of toxins (those which must gain access to the target cell cytosol), binding must be followed by translocation of at least a portion of the molecule through the bilayer into the cytosol. In many cases this requires endocytosis of the bound toxin in either coated or uncoated

vesicles. Often the low pH of the endosome is required to activate toxins in this group. Other enzymatic toxins may be able to translocate their active domains directly across the plasma membrane, although in no case has endocytosis been entirely ruled out.

In the second group of membrane penetrating bacterial protein toxins, activation results either in the insertion of a pore structure into the plasma membrane, destroying the integrity of the bilayer and disrupting the cell's osmotic balance, or in the stimulation of a specific signal transduction pathway via the interaction with a transmembrane receptor.

Toxins that are Known to be Enzymes

A general characteristic of the enzymatic toxins is their A-B composition. The A domain or subunit contains the catalytic activity while the B portion is responsible for receptor binding and membrane translocation. In many cases, the entire toxin is the product of a single gene, and the A and B portions represent domains within the nascent polypeptide which are often ultimately separated by proteolytic processing. In other toxins the B-portion is a homo- or a hetero-oligomer of subunit proteins which is assembled before being released into the extracellular milieu.

Toxins that are ADP-ribosyltransferases

One major sub-division of the A-B toxins contains those which modify specific proteins in the target cell by adding an adenosine diphosphate ribose group (ADP-R), derived from nicotinamide adenine dinucleotide (NAD^+), to one or more of the protein's amino acid side chains.

Diphtheria toxin

The Gram positive bacterium *Corynebacterium diphtheriae* is still endemic in large parts of the developing world. It is responsible for diarrheal disease (diphtheria) in millions of people. An important virulence factor for this organism is the 58 kDa protein diphtheria toxin (DT) which is encoded by a lysogenic phage (Greenfield *et al.,* 1983). The toxin is secreted as a single polypeptide chain that is subsequently cleaved into two fragments (Murphy, 1985) which remain connected via a disulfide bridge (Cys-186 to 201). After binding to a protein receptor on target cells the toxin is endocytosed. The N-terminal A portion (21kDa) is translocated across the endosomal membrane and released by reduction of the disulphide bond into the target cell cytosol where it ADP-ribosylates the diphthamide residue of elongation factor-2 (EF-2), thereby inactivating the protein (van Ness *et al.,* 1980). Unable to synthesize any new protein, the cell quickly dies. The larger B portion (37 kDa) contains both the receptor binding domain and structures essential for the membrane translocation of the A domain into the cytosol (London, 1992).

The toxin binds to its target cells (enterocytes) via the heparin-binding epidermal growth factor-like growth factor precursor (Naglich *et al.,* 1992). The reason why a growth factor is expressed as a surface protein is controversial, but it has been demonstrated that many growth factors can be found in a membrane-bound state from which they can subsequently be released by proteolysis, or interact with neighboring cells directly (Naglich *et al.,* 1992; Ono *et al.*, 1994). Rolf and Eidels (1993) cleaved DT into

smaller polypeptide fragments, and were able to localize the DT receptor-binding domain to the C-terminal 54 amino acids of the toxin. Based on the three dimensional structure, determined from x-ray crystallographic data, this region corresponds to three β-strands exposed on the surface of diphtheria toxin (Choe *et al.*, 1992). It is proposed that protein-protein interactions are formed between a loop in this B-domain peptide (including Lys-516 and Phe-530) of DT and the extracellular N-terminal portion of the membrane-spanning receptor (Collier *et al.,* unpublished).

The B-domain of DT is able to form transmembrane ion-channels at low pH (Kagan *et al.*, 1981; Madshus *et al.*, 1994). The translocation portion of the B-domain contains nine α-helices, at least two of which show the classical pattern of amphipathicity associated with many membrane channel-forming helices (Greenfield *et al.,* 1983; Eisenberg, 1984). Choe *et al.* (1992) have suggested that one or more pairs of these helices could penetrate the membrane under the stimulus of low endosomal pH, since the acidic residues in the connecting loops would be neutralized by protonation in the endosomal environment. Although these channels are not thought to transport the A-domain through the membrane, it seems likely that they form a part, or are a remnant of, the translocation machinery.

Cholera toxin and *Escherichia coli* heat labile enterotoxin

The Gram negative bacteria *Vibrio cholerae* secretes a potent enzymatic toxin into the intestine of the host organism which penetrates the cells lining the intesinal tract and ultimately disrupts ion and water transport across the enteric cell membrane. Cholera

toxin (CT) is made up of two parts, a single A subunit (27 kDA) and a pentameric ring of B subunits (11.6 kDa each). A single copy of the gene for each subunit type (*ctxA* and *ctxB* for the A and B subunit respectively) is found together in an operon that is duplicated in many strains of *Vibrio cholerae* (Yamamoto *et al.,* 1987; Moseley and Falkow, 1980). Each protein is transcribed under the control of its own promoter, thus enabling the cell to synthesize an efficient 1 to 5 ratio of A to B subunits. The subunits are assembled into the hexameric toxin in the periplasm before being secreted across the outer membrane and into the gastrointestinal tract inhabited by the bacteria. The A subunit is divided into two domains: A_1 (22 kDA) contains ADP-ribosyltransferase activity and A_2 (5 kDa) links A_1 to the B pentamer and may assist in membrane translocation. In the nascent toxin, these two domains are found as a continuous polypeptide chain joined by a flexible loop which is proteolytically nicked, most likely by host proteases on the surface of the target cell or in the intestine, leaving only a disulfide bridge linking A_1 to A_2. In order for the toxin to be activated, this disulfide bond is ultimately reduced to release the A_1 subunit into the cytosol of the enterocyte (Mekalanos *et al.*, 1983; Sixma *et al.*, 1991).

The toxin binds to the basolateral surface of enterocytes via an interaction between the B-subunit and the oligosaccharide moiety of ganglioside G_{M1}. Van Heyningen *et al.* (1971) found that ganglioside preparations could inhibit CT activity on cells. Cuatrecasas (1973) went on to demonstrate that G_{M1} was the most potent CT inhibitor of the gangliosides, after comparing the CT binding activity of G_{M1} with a wide selection of glycolipids, glycoproteins and oligosaccharides. The specific nature of

binding to G_{M1} was confirmed using a G_{M1}-deficient cell line, murine NCTC-2071A fibroblasts, which is insensitive to CT. When these cells were incubated with G_{M1}, the ganglioside incorporated into the plasma membrane and the cells became CT sensitive (Moss *et al.*, 1976). By using both radiolabeled gangliosides and radioiodinated CT, it was possible to determine the stoichiometry of cholera toxin binding to G_{M1} molecules incorporated into the membrane. Fishman (1986) found that between 5 and 7 G_{M1} gangliosides were bound by each CT molecule. Therefore, it seems that all five subunits of the B-pentamer have a receptor-binding site, and that all of these can be occupied by a G_{M1} on the target cell membrane (Miller-Podraza, 1982). This could suggest a model for CT action in which all five B-subunits must bind to ganglioside receptor before the A_1 subunit can be translocated to the cytosol and be active. This multivalent binding is facilitated by the high mobility of G_{M1} in the membrane (Spiegel *et al.*, 1984).

Much of what is known about the structure of cholera toxin has been based on its high degree of homology with the *Escherichia coli* heat-labile enterotoxin (LT). LT has been crystallized and the X-ray structure has been determined of both the holotoxin and the B-pentamer bound to lactose (thought to be a good analogue for the cellular G_{M1} receptor; Sixma *et al.*, 1991; 1992). When the structure of the LT/CT ADP-ribosylation domain was compared with those of other enterotoxins, significant structural homology with the ADP-ribosylation domains of diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A (which ADP-ribosylates EF-2) was noted (Sixma *et al.*, 1991). LT was shown to have lower receptor binding specificity than CT. Whereas CT has a high affinity for G_{M1} and does not seem to readily bind to alternative membrane components

(for example other gangliosides), LT has been shown to bind a broader spectrum of plasma membrane components, including G_{M1} and other gangliosides (Griffiths *et al.*, 1986).

Once bound to the membrane, the active portion of the toxin (A_1) must gain access to the cytosol in order to ADP-ribosylate an arginine residue on its target protein ($G_{s\alpha}$; Cassel and Selinger, 1977). It is unclear how this is achieved, but it seems that endocytosis of the bound CT is not required, in contrast to the mechanism described for diphtheria toxin. Sixma *et al.* (1992) suggested that the B pentamer could form a membrane channel through which the enzymatic subunit could pass into the cytosol. Indeed, it appears that the B subunit alone is able to form ionic channels in planar lipid bilayers (Krasilnikov *et al.*, 1991). However, there is evidence that insertion of the B subunit into the bilayer is not necessary for A_1 subunit translocation (Wisnieski *et al.* 1981; Ribi *et al.*, 1988). Fishman (1990) proposed a model in which the multivalent binding of the B pentamer to G_{M1} on the target cell would result in localized perturbation of the membrane, and induce a conformational change in the holotoxin which would cause the insertion of the A subunit into the membrane. The A_1 ADP-ribosyltransferase domain would subsequently be activated and released into the cytosol by reduction of the disulfide bond linking it to A_2, either by cytoplasmic glutathione or a membrane protein.

The ADP-ribosylation of the signaling protein $G_{s\alpha}$ by CT activates it, and activated $G_{s\alpha}$ in turn stimulates adenylate cyclase, elevating cellular cyclic adenosine monophosphate (cAMP) levels (Northup *et al.*, 1980). The increased level of cAMP causes the opening of ion channels in the cell membrane resulting in the loss of chloride

and water from the cell into the gastrointestinal tract, which is subsequently manifested as diarrhea. $G_{s\alpha}$ is normally stimulated by the binding of guanosine triphosphate (GTP) in response to extracellular stimuli, for example hormone binding to a trans-membrane receptor. The protein subsequently deactivates itself by means of an inherent GTPase activity (Gilman, 1987). In the ADP-ribosylated protein however, the GTPase activity is inhibited, so that $G_{s\alpha}$ is not easily inactivated and persistent overstimulation of adenylate cyclase results (Cassel and Selinger, 1977). The ADP-ribosylation activity of cholera toxin is not entirely specific to $G_{s\alpha}$: there are reports of CT ADP-ribosylating a variety of different substrates, including ADP-ribosylation of the A_1 subunit itself (Trempel *et al.*, 1977; Moss *et al.*, 1980; Northup *et al.*, 1980). The *in vivo* consequences and significance of CT ADP-ribosylation of these alternative substrates remains to be fully investigated.

A family of guanine-nucleotide binding proteins that can stimulate CT ADP-ribosylation has been identified (Moss and Vaughan, 1991). These ADP-ribosylation factors or ARFs appear to be ubiquitous in eukaryotic cells and probably play a role in the normal cellular ADP-ribosylation cycle which will be discussed later in this introduction.

Other enzymatic toxins

***Clostridial* neurotoxins**

The Gram positive *Clostridial* species produce a number of highly toxic neurotoxins in addition to a number of other toxins and virulence factors. The botulinum neurotoxins (BoNT) are the most potent toxins known (Gill, 1982). Less than 1 ng per kg body weight is lethal to humans. Seven different serotypes (A to G) of BoNT have been identified, which vary in their specific cellular targets giving rise to differing toxicities and cell line specificities (Blasi, 1993; Schiavo *et al.*, 1992; 1993a; 1993b). Although initially characterized from *Clostridium botulinum*, a few of the BoNT have also been found to be secreted by other *Clostridial* species (Hall *et al.*, 1985, McCroskey *et al.*, 1985). Tetanus toxin (TeT), which is produced exclusively by *C. tetani,* differs from the BoNT in its site of action. Whereas all seven of the BoNT act to inhibit acetylcholine (ACh) release at the neuromuscular junction, TeT inhibits neurotransmitter release at the inhibitory interneurons in the spinal cord which it reaches via retrograde axonal transport from neuromuscular junctions (Schwab, 1979).

Both TeT and BoNT are released from the *Clostridial* cell as 150 kDa single chain proteins that are subsequently cleaved at a surface loop to yield 100 kDa heavy chains (H) and 50 kDa light chains (L) which remain connected via disulfide bonds (Montecucco *et al.*, 1988). The amino acid sequence of TeT and several of the BoNT serotypes have been determined and comparison of these sequences has revealed 30 to 50% overall identity between them. Certain sequences and motifs show almost complete

conservation and many of these are proposed to have essential roles in the toxins' functions (Whelan *et al.*, 1992). As with the A-B toxins described above, the larger H subunit carries the receptor binding and translocation domains of the toxin, while the L-chain carries the enzymatic active site (Helting, 1977; Helting and Zwisler, 1977; Bandyopadhay *et al.*, 1987).

Gangliosides appear to play an important role in the binding of TeT and BoNT to the target cell. For the more clinically important BoNT serotypes (A, B, and E) and TeT, the specific gangliosides to which these toxins bind with highest affinity have been investigated (Morris *et al.*, 1980; Takamizara *et al.*, 1986; Kozaki *et al.*, 1987a; Montecucco *et al.*, 1988; Lazaroici *et al.*, 1987; Kitamura *et al.*, 1980). The high degree of specificity of the BoNT and TeT for the neuronal synaptic membrane suggests that something more unique to this membrane than gangliosides may be involved in toxin binding (Nishiki *et al.*, 1994). In 1991, Ogasawa *et al.* demonstrated that the binding of BoNT serotype B to mouse brain synaptosome membranes could be reduced by treatment of the membranes with protease, neuraminidase, or phospholipase C. These researchers went on to identify a 58 kDa protein in the mouse synaptosome membrane as a putative BoNT-B receptor (Nishiki *et al.*, 1994). This protein has amino acid sequence identity and antibody cross-reactivity with synaptotagmin, a known integral membrane protein of the synaptic vesicle which is unique to neuronal membranes (Perrin *et al.* 1990), and is thought to be able to interact with gangliosides. It seems likely then, at least for BoNT-B, that the cellular receptor for the toxin is a ganglioside / protein complex, since depletion of either component reduces toxin binding (Perrin *et al.* 1990).

Once bound to the synaptic membrane, BoNT and TeT are internalized and become activated in the acidic environment of the endosome (Roa and Bouquet 1985; Boquet *et al.* 1984). The translocation step for each could resemble that of diphtheria toxin since it has been discovered that, like DT, the heavy chains of both BoNT-B and TeT form trans-membrane ion channels which may play a vital role in the translocation of the active L-chain of these toxins to the cytosol (Hoch *et al.* 1985).

In recent years, TeT and BoNT have been shown to be zinc proteases which cleave specific vesicular protein targets in the neurons, inhibiting the fusion of neurotransmitter containing vesicles with the presynaptic membrane and hence blocking neurotransmitter release (Vallee and Auld, 1990; Schiavo *et al.*, 1992a; 1992b; 1992c; 1993a; 1993b; Blasi *et al.* 1993; Montecucco 1994).

Anthrax toxin

The anthrax toxin of *Bacillus anthracis* consists of three different proteins, protective antigen (83 kDa), edema factor (89 kDa) and lethal factor (83 kDa). The protective antigen (PA) is analogous to the B-domain of the A-B toxins described above, being responsible for binding to the receptor on target cells and containing a vital component of the membrane translocation machinery for the active domain. Subsequent to binding to an unknown receptor found on a spectrum of target cells, PA is cleaved by a cell surface protease which removes a 20 kDa fragment from the free N-terminus of the protein (Singh *et al.*, 1991; Klimpel *et al.*, 1992), leaving a 63 kDa PA fragment still bound to the target cell membrane. Removal of the 20 kDa fragment exposes a high-

affinity binding site on the 63 kDa PA for either the edema factor (EF) or the lethal factor (LF). Either of these proteins can now bind to the 63 kDa PA via an amino-terminal PA-binding domain that they both contain (Novak *et al.*, 1992; Arora and Leppla, 1993; Milne *et al.*, 1995).

All three toxin genes are encoded on a large plasmid (114 MDa), pXO1, found in all virulent strains of *B. anthracis* (Mikesell *et al.*, 1983). The arrangement and regulation of the toxin genes on pXO1 is unknown, but PA is likely to be synthesized in excess relative to EF or LF since neither of these can act without PA binding to, and being processed on, the target cell surface. Friedlander (1986) has shown in *in vitro* experiments using purified toxin components that a ten-fold excess of PA over LF (10^{-2} and 10^{-3} μg/ml respectively) is required for the toxin to be effective.

After the EF or LF are bound to processed 63 kDa PA immobilized on the target cell surface, the toxin complex is endocytosed and the active subunit translocated into the cytosol from the endocytotic vesicle (Friedlander, 1986). Little is know about the translocation mechanism, but it may involve the oligomerization of a number of PA subunits which can insert into the bilayer to form a channel across which the enzymatic subunit could be translocated (Blaustein *et al.*, 1989; Milne *et al.*, 1995; Collier, unpublished).

The edema factor has been characterized as a calmodulin-dependent adenylate cyclase (Leppla, 1982) that is able to function in any cell which it successfully penetrates. The resulting elevated cAMP levels, as with cholera toxin, lead to chloride and water loss from the affected cells, which in *B. anthracis* infection cause localized swelling and

edema (Stanley, 1961). The action of the edema toxin upon polymorphonuclear neutrophils (PMNs) is of particular importance to the pathogenicity of *B. anthracis* (O'Brien *et al.*, 1985). Exposure of cultured PMNs to a combination of PA and EF inhibits their ability to phagocytose *B. anthracis* cells, indicating that edema toxin is particularly important in the early stages of infection in protecting the bacteria from the initial immune response (O'Brien *et al.*, 1985).

The mechanism of action of the lethal factor still remains uncertain, but there is some recent evidence to suggest that it may, like the *Clostridial* neurotoxins, be a zinc metalloprotease. The cellular target for this activity is unknown but apparently it is unique to macrophages, since lethal toxin has been found to be inert in all cell lines tested except macrophages, even when internalized (Hanna *et al.*, 1993; 1995; Friedlander, 1986; Singh *et al.*, 1989). The ultimately lethal response evoked by the action of the lethal toxin in the macrophage involves an oxidative burst. Hanna *et al.* (1995) demonstrated that murine and human macrophages which lack the ability to produce reactive oxygen intermediates are less sensitive to anthrax lethal toxin, whereas a murine macrophage cell line with increased oxidative burst potential was more sensitive to lethal toxin than wild type macrophages. In addition, they found that mice depleted of macrophages were resistant to lethal toxin but that sensitivity could be restored by the injection of macrophages (Hanna *et al.*, 1993). It remains to be determined how the activated lethal factor achieves these effects within the cell and how this is related to the sudden death associated with *B. anthracis* infection.

Membrane Located Toxins

The other group of toxins contains those proteins which exert their toxic effects without the need to pass beyond the target cell's plasma membrane. These toxins can be further divided into two groups. One contains the cytolysins, which form structures that insert into the plasma membrane forming holes or pores through which solutes and water can freely pass, thus destroying the cell's osmotic regulation and usually leading to lysis. The second contains a small group of toxins which seem to bind hormone receptors on the cell surface and activate signal transduction pathways leading to cellular dysfunction (Thelestam and Florin, 1994). Since this thesis is concerned with membrane inserting toxins, only members of the first group are described further.

Bacterial protein toxins that are cytolytic

Cytolytic bacterial protein toxins have been identified and characterized to differing extents in a wide range of both Gram positive and Gram negative bacteria. The feature common to all these proteins is the ability to form aqueous transmembrane pores, usually involving the oligomerization of a number of the toxin subunits. The formation of such pores requires that these proteins possess amphipathic sequences, with hydrophobic surfaces which penetrate and make contact with the membrane interior.

The RTX toxins

As early as 1903 it was observed that some *E. coli* cultures secreted a factor which could lyse erythrocytes. In 1963 the soluble factor was isolated and named α-hemolysin

by Smith (1963) to differentiate it from a cell-associated factor which he named β-hemolysin (reviewed in Cavalieri *et al.*, 1984). α-hemolysin has since been shown to be one member of a large family of Gram negative bacterial protein toxins which share a number of common structural and functional features. These toxins were named the RTX (repeats in toxin) toxins as each family member contains a nine amino acid repeat sequences near the C-terminus (reviewed by Welch, 1991). Other members of the RTX family, not all of which are hemolytic, have been identified in a number of bacterial species, including *Pasteurella hemolytica* (leukotoxin; Strathdee and Lo, 1987), *Actinobacillus pleuropneumoniae* (Apx I, II and III; Jansen *et al.*, 1994; Frey *et al.*, 1993), *Bordetella pertussis* (CyaA; Glaser *et al.*, 1988; Benz *et al.*, 1994a), *Proteus mirabilis*, *Proteus vulgaris* and *Morganella morganii* (Koronakis *et al.*, 1987; Benz *et al.*, 1994b).

The RTX toxins are mostly found encoded within the bacterial chromosome, as part of operons which also include genes for the activation and secretion of the toxins (Welch 1991; Frey *et al.*, 1993; Jansen *et al.*, 1994; Koronakis 1987). They utilize a novel mechanism for secretion involving the protein products of other genes from the same operon, which enables them to bypass the periplasm of the Gram negative cells in which they are produced (reviewed by Hughes *et al.*, 1992).

Although it has been known for some time that the protein product of one of the other genes in the toxin operon (*hly C* in *E. coli*) is essential for activation, it is only recently that the enzymatic function of this protein has been determined (Hardie *et al.*, 1991; Issartel *et al.*, 1991). Hly C fatty acylates *E. coli* α-hemolysin at two sites prior to

secretion and this modification enables the toxin to insert into target cell membranes. The activation proteins of other RTX toxins possess similar acylating activities (Stanley *et al.*, 1994). A variety of fatty acid types can be added to the protein, and it appears that the acylation enzymes for each RTX toxin member show different preferences for the fatty acid used. In addition, the specific activity of α-hemolysin has been shown to be in part dependent upon the fatty acid species attached. This has led to the proposal that the fatty acylation of the toxin plays an important part in target cell binding (Hughes *et al.*, 1992). This idea is not unprecedented; Spaink *et al.*, (1991) demonstrated that the host specificity of *Rhizobium* is determined by a specialized fatty acid moiety produced by the bacteria.

The variation in target cell specificity among members of the RTX toxin family has led to the conclusion that each toxin must bind to a specific receptor on its target eukaryotic cell. None of these receptors has yet been characterized, but recently a great deal has been learned about the receptor-binding domain of the *E. coli* toxin, and how the receptor specificities of the individual RTX toxins may be determined. Boehm *et al.* (1990) demonstrated that calcium is essential for receptor binding and lytic activity of the *E. coli* α-hemolysin. The Ca^{2+} is bound by the RTX repeats in the C-terminal portion of each family member, and is required for the toxin to adopt an active conformation (Ludwig *et al.*, 1993). If *E. coli* are grown in calcium-free media, the secreted α-hemolysin is inactive, but the addition of Ca^{2+} results in hemolytic activity. The Ca^{2+} is irreversibly bound to the toxin. Even if a chelating agent, such as EGTA [ethylene glycol-bis(β-aminoethyl ether)-N, N, N',N'-tetraacetic acid], which will bind free Ca^{2+}, is

added in excess to the initial Ca^{2+} concentration, activity cannot be fully blocked (Boehm *et al.*, 1990). The acylation domain is immediately adjacent to the Ca^{2+} binding repeats, further supporting the idea that it plays a role in target cell binding.

Hemolysis results from pore formation by toxin bound to the target cell membrane. Recently, the functional pore has been demonstrated to comprise a multimer of the approximately 100 kDa RTX toxin monomers (Ludwig *et al.*, 1993). It is uncertain whether insertion into the bilayer precedes aggregation, or whether the pore forms on the membrane surface prior to insertion, but it seems that a single α-hemolysin is able to insert unilaterally into a membrane (Ludwig *et al.*, 1991). Ludwig *et al.* (1991) used several mutants of *E. coli* α-hemolysin to investigate which part of the 110 kDa toxin sequence was responsible for pore formation. They proposed a structure for the pore-forming domain in which eight α-helices are formed, four hydrophobic and four amphipathic, by an N-terminal portion of the toxin sequence spanning about 250 amino acids. Menestrina *et al.* (1994) have proposed a similar model based on the comparison of the amino acid sequences of six RTX toxins. They suggest that each monomer can form as many as ten amphipathic α-helices from the same conserved N-terminal domain.

The thiol-activated toxins

A number of Gram positive bacteria produce toxins that share many structural and functional properties. All of these toxins have a free cysteine residue and until recently it was believed that the reduction of this residue was required for toxin activation. This led to the naming of this group of at least 15 toxins as the thiol-activated toxins, or

sometimes the oxygen-labile toxins, since oxidation led to inactivation of the toxins (Boulnois *et al.*, 1991). All of these toxins are secreted as monomers of 50 to 70 kDa (Alouf *et al.* 1984). Although streptolysin O, produced by most pathogenic *Streptococci*, is the prototypical member, several other members of this family of toxins have also been studied in some depth. At least five of the toxins (pneumolysin, streptolysin O, listeriolysin, perfringolysin O and alveolysin) have been cloned and sequenced (Walker *et al.*, 1987; Kehoe *et al.*, 1987; Tweten, 1988; Mengaud *et al.*, 1988; Geoffroy *et al.*, 1990). The sequences revealed that the toxins lack extended hydrophobic stretches, indicating that any transmembrane structures formed by the toxin will most likely have an amphipathic nature (Boulnois *et al.*, 1991).

Cholesterol has been proposed as the cellular receptor for the thiol-activated toxins, but conclusive evidence for this hypothesis is lacking (de Kruijff, 1990). It is clear from the work of numerous investigators that cholesterol plays an essential role in the cytolytic activity of these toxins (Alouf *et al.*, 1984; Bhakdi *et al.*, 1985; Cowell *et al.*, 1978; de Kruijff, 1990; Iwamoto *et al.*, 1993; Owen *et al.*, 1994; Prigent and Alouf, 1976). It has been shown that free cholesterol inhibits hemolysis (Saunders et al 1989), but this does not rule out a role for cholesterol in a subsequent step, rather than in target cell binding. Each of these proteins has only a single cysteine residue which is located within a conserved sequence (-ECTGLAWEWWR-) found near the C-terminus (Boulnois *et al.*, 1991). This sequence may be important for binding to membrane cholesterol, but it is not essential for binding to target cell membranes. de Kruijff (1990) proposed that the binding of cholesterol by this domain changes the toxin's orientation,

stimulating membrane penetration (a mechanism also proposed for the polypeptide antibiotic Gramicidin A, which has a homologous tryptophan rich sequence). Once bound, these toxins are able to form large oligomers (containing as many as 26 monomers for pneumolysin) which are thought to insert into the membrane forming lesions up to 35 nm in diameter and destroying the cell's osmotic balance (Bhakdi *et al.*, 1985; Morgan *et al.*, 1994; Sekiya *et al.*, 1993).

Pseudomonas aeruginosa **cytotoxin**

The Gram negative bacteria *Pseudomonas aeruginosa* is an opportunistic pathogen which typically infects only immunocompromised patients. In addition to a well-characterized exotoxin (exotoxin A) which has both structural and functional similarities with cholera toxin (Allured *et al.*, 1986), and a number of less well characterized toxins (Lory and Tai, 1985), the bacteria produces a cytolytic protein toxin. The gene for the 28 kDa cytotoxin has been found on a temperate phage which integrates at a specific site on the host bacterial chromosome. The toxin is synthesized as a 31 kDa precursor that remains bound to the cell's outer membrane until released into the intracellular environment as the 28 kDa form by proteolysis, perhaps only as the bacterial cell is itself lysed (Hayashi, 1989).

After binding to target cells, the 28 kDa soluble form of the toxin is thought to assemble into oligomeric structures, which insert into the membrane to form water-filled ion channels of 1 nm internal diameter (Suttorp *et al.* 1985). Recently the toxin was shown to bind to a glycoprotein on rabbit erythrocytes which appears to be a member of

the family of water channel proteins including human CHIP-28 (Lutz *et al.*, 1993; Jungblut *et al.*, 1992). A combination of techniques was used to study binding, including probing various preparations of rabbit erythrocyte proteins (immobilized on nitrocellulose membranes after separation by SDS-PAGE) with ^{125}I-labeled cytotoxin, and binding of the radiolabeled toxin to whole rabbit erythrocyte membranes directly. Toxin binding was unaffected by enzymatic removal of N-linked glycans and by limited proteolysis (Lutz *et al.*, 1993). The toxin was found to bind specifically to CHIP-28 when either purified human CHIP-28 or a partially purified fraction containing CHIP-28 protein was run on SDS-PAGE, transferred to nitrocellulose and probed with the radiolabeled toxin.

The discovery that the toxin receptor in erythrocytes is itself an endogenous water channel opens up the possibility that the toxin may act by stimulating the opening of this channel thereby obviating the need to form a channel of its own. Since there is only limited evidence for pore formation by this toxin, and no oligomeric structures have been well characterized (Hayashi *et al.*, 1990), this possibility cannot be eliminated (Wilmsen *et al.*, 1994). If it were true then it is possible that the toxin could still act when in its membrane-bound form if the bacterial cell were in proximity to the target cell.

***Staphylococcus aureus* α-toxin**

Staphylococcus aureus is among the most common bacterial causes of infection and disease in humans. It is implicated in a broad range of ailments, from harmless boils to lethal septicemias (Bhakdi and Tranum-Jensen, 1991). Although it has become clear that different *S. aureus* strains can produce several toxins (including toxic shock

syndrome toxin-1, which is thought to act as a pseudohormone), the α-toxin is by far the most thoroughly studied.

The α-toxin gene has been cloned and its location mapped on the bacterial chromosome, although it may be carried between bacteria on a lysogenic phage (Brown and Patee, 1980; Patee, 1986). Although most studies make use of the hemolytic properties of α-toxin, this may not be its major mode of action in all disease states. The toxin can have profound effects on a number of cell lines, including macrophages, epithelia, fibroblasts, smooth muscle and mast cells (Bhakdi and Tranum-Jensen, 1991; Kato and Noda, 1989; Bhakdi *et al.*, 1988; 1989; Suttorp *et al.*, 1985; McGee *et al.*, 1983; Blomqvist and Thelestam, 1986; Jonas *et al.*, 1994; Walev *et al.*, 1993), many of which are not lysed.

The sensitivity of erythrocytes from different species to hemolysis by α-toxin is known to vary greatly. Rabbit erythrocytes are the most sensitive, being about 150 fold more sensitive than human cells, which are among the least sensitive (Bernheimer *et al.*, 1965). Cassidy and Harshman (1976) studied the binding of ^{125}I-labeled α-toxin to rabbit and human erythrocytes. Their results indicated that whereas the rabbit cell has approximately 5,000 copies of a high-affinity receptor for α-toxin to which the toxin binds specifically and irreversibly, the human cell has no detectable high-affinity binding sites for α-toxin. Binding of *S. aureus* α-toxin to target cells has been proposed to proceed by two different mechanisms. At low concentrations the toxin binds to a limited number of high affinity receptors found on the surface of susceptible cells, whereas at higher concentrations (greater than 1μM) the toxin can adsorb non-specifically onto any

plasma membranes (Hildebrand *et al.*, 1991; Forti and Menestrina, 1989; Bhakdi *et al.*, 1983).

The specific receptor for *S. aureus* α-toxin from rabbit erythrocytes has not yet been identified. Although Maharaj and Fackrell (1980) proposed that it was band 3, the erythrocyte anion channel, their results have not been confirmed and are at odds with the results of other research. They showed that the sensitivity of rabbit erythrocytes to α-toxin could be reduced by pre-treatment of the cells with pronase, an enzyme known to degrade band 3, and that pre-incubation of cells with lectins known to bind band 3 could similarly inhibit toxic activity. However, this conclusion has been shown to be premature in the light of other results. Erythrocyte membranes display a large number of proteins, many as yet uncharacterized, some of which may possess the same pronase sensitivity and lectin binding pattern as band 3 (Dandzu *et al.*, 1985; Schnitzer *et al.*, 1992; reviewed by Krotkiewski, 1988). Also, the receptor is present at only 5,000 copies per cell, whereas band 3 has 100 times this many copies (Cassidy and Harshman, 1976; Hildebrand *et al.*, 1991). Finally, the ubiquity of band 3 in the membranes of erythrocytes of all species seems at odds with the high species specificity of α-toxin binding. All these results together suggest that the conclusion drawn by Maharaj and Fackrell (1980) may have been incorrect.

Regardless of how the toxin is held on the membrane, the subsequent activation steps leading to cell lysis appear to be the same. The hemolytic mechanism for α-toxin is thought to depend upon the ability of the 33 kDa monomers to form oligomeric pores of 1-2 nm internal diameter (Füssel *et al.*, 1981). Although the amino acid sequence of the

protein revealed that it does not contain any long stretches of hydrophobic amino acids characteristic of transmembrane proteins, structural studies have revealed an abundance of amphiphilic β-sheet structure (Tobkes *et al.*, 1985). Oligomerization of the monomers is thought to lead to the formation of a β-barrel, where each monomer contributes a number of β-strands to an oligomeric ring, oriented with the hydrophobic residues facing out towards the membrane lipids, and their hydrophilic residues all facing inwards to form a water-filled pore (Bhakdi and Tranum-Jensen, 1991; Menestrina *et al.*, 1992; Harshman *et al.*, 1989). Oligomerization has been shown to be required for membrane insertion (Harshman *et al.*, 1989). Although most early evidence pointed towards a hexamer as representing the functional oligomer, more recent results suggest that the toxin can also adopt a heptameric conformation, but the *in vivo* importance of this form is not understood (Gouaux *et al.*, 1994). Binding to the membrane is thought to induce a conformational shift in the monomer which promotes oligomer formation, and ultimately pore formation (Harshman *et al.*, 1989). Oligomerization is also promoted on the membrane by the effective concentration of the toxin on the cell surface as compared with the surrounding solution. Cell-bound monomer-receptor complexes move around in the plane of the membrane and come into contact with each other, enabling multimer formation (Tomita *et al.*, 1992).

Clostridium septicum α-toxin

Clostridium septicum, a causative agent of gas gangrene, produces a number of toxins. The mechanism, structure and role in pathogenicity of one of these, the α-toxin,

have recently been investigated. Ballard *et al.* (1995) cloned the gene for the lethal toxin (α-toxin) of *C. septicum* that encodes a 48 kDa protein. They found significant homology (27% identity /72% similarity over a 387 aa stretch of sequence) with the *Aeromonas hydrophila* hemolysin, aerolysin. In addition, earlier characterization of the purified α-toxin had indicated that it shares many mechanistic characteristics with aerolysin (Ballard *et al.*, 1992).

The α-toxin is encoded genomically and is secreted, like aerolysin, as a protoxin which must be activated by proteolytic cleavage at a site approximately 70 amino acids from the C-terminus (Ballard *et al.*, 1993). The activated toxin forms oligomeric aggregates of approximately 230 kDa and generates ion channels in bilayers with a pore size estimated to be 1.3 to 1.6 nm in diameter (Ballard *et al.*, 1993).

Nothing is currently known about the receptor for *C. septicum* α-toxin, however, considering the many similarities between this toxin and *A. hydrophila* aerolysin, the recent identification of a receptor for the latter toxin (Cowell and Buckley, unpublished) may lead to a breakthrough in the identification of a receptor for the α-toxin.

Aerolysin of *Aeromonas hydrophila*

The Gram negative *Aeromonads* have been implicated in a range of disease states, from furunculosis, probably the most widespread and destructive bacterial diseases of *Salmonid* fish, to diarrheal disease and wound infections in man (Janda, 1991). Although *A. salmonicida* has been most commonly diagnosed as the causative agent of furunculosis, there is evidence that *A. hydrophila* can also cause a number of severe

disorders in commercially important fish species (Allan and Stevenson, 1981). Human *Aeromonas* infections had been thought to affect only immunocompromised patients but there are a number of case studies which identify *A. hydrophila* as the primary pathogens in wound infections in healthy individuals, usually as a result of contact with polluted water (Hanson *et al.*, 1977; Davis *et al.*, 1978; Joseph *et al.*, 1979). In addition, there is increasing evidence from a large number of diarrheal patients that *A. hydrophila* can act as a gastrointestinal pathogen, especially in young children (Janda, 1991; Kuijper *et al.*, 1989).

A. hydrophila secretes a number of proteins into the extracellular environment, including a glycerophospholipid-cholesterol acyltransferase (Robertson *et al.*, 1992; MacIntyre and Buckley, 1978), proteases (Loewy *et al.*, 1993; Rodriguez *et al.*, 1992; Leung and Stevenson, 1988a; 1988b), amylases (Chang *et al.*, 1993), enterotoxins (Ljungh *et al.*, 1982) and several hemolysins (Kozaki *et al.*, 1987a; Asao *et al.*, 1986; Bloch and Monteil, 1989; Howard and Buckley, 1985a; Ljungh *et al.*, 1981; Thelestam and Ljungh, 1981).

The best characterized of these protein products is the hemolysin, aerolysin, first described by Bernheimer and Avigad in 1974. The gene for aerolysin has been cloned from the *A. hydrophila* chromosome (Howard and Buckley, 1986; Chakraborty *et al.*, 1986), and the nucleotide sequence determined (Howard *et al.*, 1987). The gene encodes a 54 kDa protein with a 23 amino acid signal sequence, which in Gram negative bacteria targets the protein to the periplasm. The signal sequence is removed concomitant with translocation across the inner membrane (Howard and Buckley, 1985a), yielding

proaerolysin. The protoxin apparently folds and dimerizes in the periplasm to assume its mature conformation before being secreted across the outer membrane (Hardie *et al.*, 1995). Proaerolysin is activated by proteolytic nicking at a site around 50 amino acid residues upstream of the C-terminus (Howard and Buckley, 1985b). The toxin has been shown to be activated by any one of a number of proteases derived from the bacterial cell or from the host organism (Garland and Buckley, 1988; van der Goot *et al.*, 1994). The peptide produced by this activation step is released and plays no further role in aerolysin toxicity (van der Goot *et al.*, 1994). Either the protoxin or the activated aerolysin dimer is able to bind to target cells, but only the proteolytically processed toxin is able to lyse cells (Garland and Buckley, 1988).

The sensitivity of erythrocytes from different species to lysis by aerolysin has been shown to vary greatly, with rat erythrocytes being the most sensitive. This was attributed to difference in phosphatidylcholine content of the target cell plasma membranes by Bernheimer *et al.* (1975). These researchers also noted that treatment of erythrocyte membranes with phospholipase C or with various proteases results in decreased aerolysin binding, suggesting that protein and phospholipids also have a role in the attachment of aerolysin to erythrocyte membranes. Howard and Buckley (1982) proposed that the receptor was a glycoprotein. They found that a glycoprotein fraction isolated from rat erythrocytes was able to inhibit aerolysin binding to intact cells, but that a total rat erythrocyte glycolipid preparation was not. Using ^{125}I-labeled aerolysin they demonstrated that pretreatment of rat erythrocytes with trypsin reduced aerolysin binding and sensitivity of the cells to lysis by the activated toxin, but chymotrypsin had no effect.

The binding results indicated that mouse erythrocytes (which exhibit the same aerolysin sensitivity as rat erythrocytes) had 9×10^5 high affinity receptors per cell for aerolysin and that binding was irreversible. Human erythrocytes, which are among the least sensitive to the toxin, contained almost no high affinity aerolysin binding sites.

In order to lyse erythrocytes, aerolysin must form oligomers which insert into the target cell membrane producing pores and disrupting the cell's osmotic balance (Howard and Buckley 1985b; Wilmsen *et al.*, 1990). Garland and Buckley (1988) showed that binding, oligomerization and insertion could be separated into three sequential steps which could be studied individually. At 4°C, aerolysin bound to the surface of rat erythrocytes and oligomerized, but could not insert into the bilayer until the temperature was raised. Protoxin bound rat erythrocytes with the same affinity as activated aerolysin but was unable to oligomerize either at 4°C or at 37°C unless treated with trypsin or another protease, confirming that activation was essential for pore formation but not for binding. When aerolysin was incubated with purified human glycophorin, the toxin aggregated and was no longer able to lyse erythrocytes. Human erythrocyte cell lines which were deficient in glycophorin were less sensitive to aerolysin. These results and Howard and Buckley's (1982) results indicating high receptor copy number and differential trypsin versus chymotrypsin sensitivity (which correspond to the protease sensitivity profile of human glycophorin), led Garland and Buckley to conclude that glycophorin and its rat analogue were aerolysin receptors.

Green and Buckley (1990) used site-directed mutagenesis to investigate the role of aerolysin's six histidine residues in receptor binding and oligomerization. They found

that a mutant protein in which the histidine at position 332 was replaced with asparagine had a reduced binding affinity for rat erythrocytes but could oligomerize normally. They concluded that this histidine residue was therefore at or near the binding site.

Once bound to the target cell membrane aerolysin is effectively concentrated, promoting oligomerization which leads to the formation of a heptameric ring structure with a central β-barrel (Wilmsen *et al.*, 1992). Two regions, both located in domain two of aerolysin (Figure 1) have been implicated in oligomerization of the toxin. Green and Buckley (1990) demonstrated that the replacement of the histidine residue at position 132 with an asparagine by site directed mutagenesis produced an aerolysin mutant that was unable to oligomerize. Mutations of tryptophans 371 or 373 to leucine, in the second region, yield aerolysin mutants which oligomerize more readily than wild type (van der Goot *et al.*, 1993). By some means the large (350 kDa) oligomeric structure penetrates the bilayer, opening up a water-filled 1 nm diameter pore between the cell interior and the extracellular environment through which ions and small solutes can freely pass. It is possible that activation of proaerolysin by cleavage of the C-terminal peptide in domain four (Figure 1) exposes a hydrophobic surface which may play a role in initiating the oligomerization of aerolysin or the insertion of the aerolysin oligomer into the membrane (van der Goot *et al.*, 1992). The resulting pore destroys the cell's osmotic balance, causing swelling of the cell and ultimately lysis (Wilmsen *et al.*, 1990). The receptor may play a role in the integration of the oligomer into the bilayer, but this hypothesis remains to be investigated. The identification of the aerolysin receptor will greatly aid this inquiry.

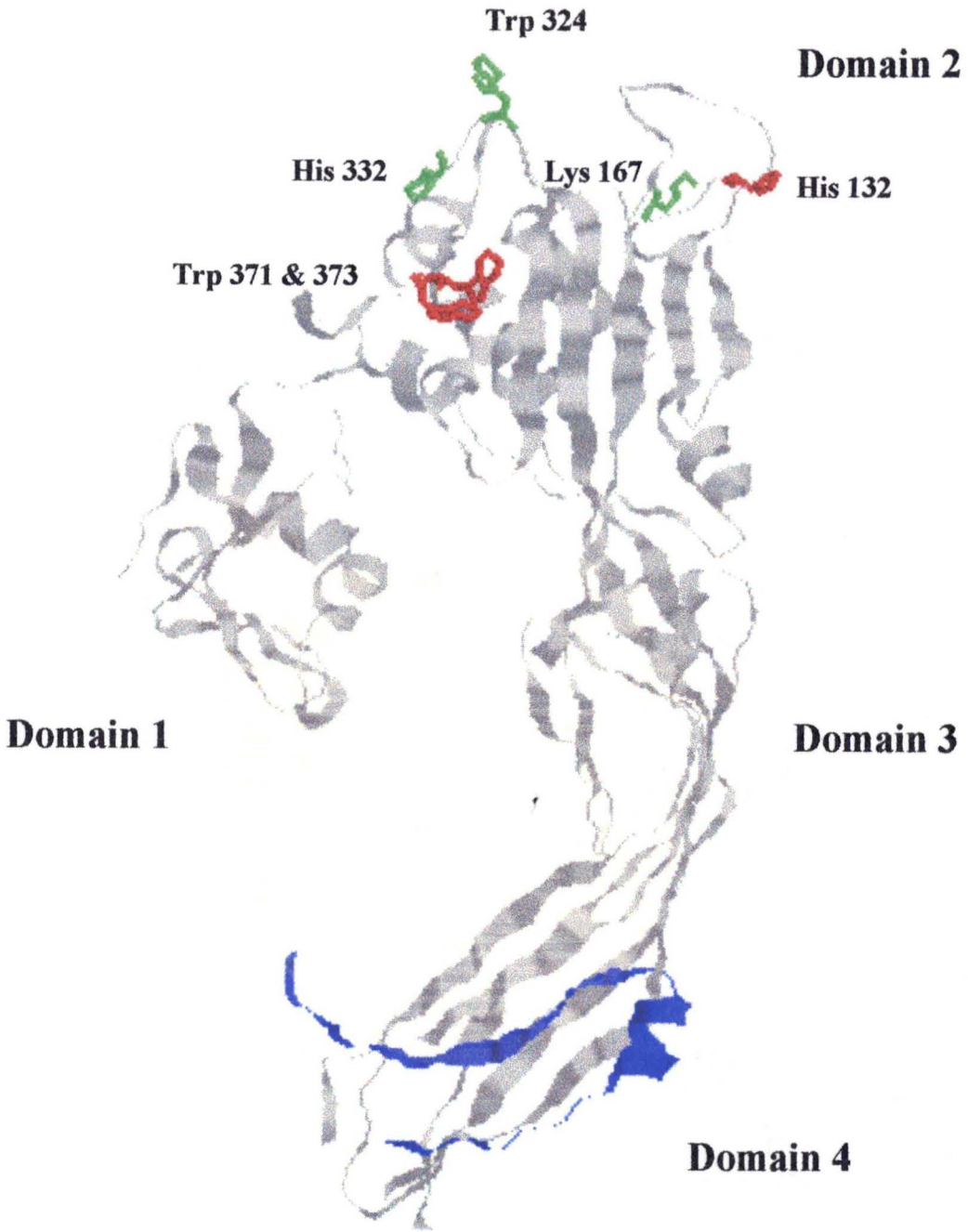

Figure 1: Molecular structure of the aerolysin monomer. Residues highlit in green are thought to be involved in receptor binding. Those residues highlit in red are involved in oligomerization. The C-terminal sequence, coloured blue, is the activation peptide which is removed by proteolytic cleavage.

Alternative Mechanisms for Bacterial Protein Toxins

For each toxin discussed above, a single, best-characterized mode of toxic activity has been given. However, there is evidence for many of these toxins that they may be able to stimulate alternative toxic effects in different cell lines or under different conditions (e.g. at low toxin concentrations) perhaps via different mechanisms. Additionally, the best-characterized mechanism associated with a toxin is not necessarily the most important effect *in vivo* but may depend on the *in vitro* assay system used. This is particularly true for cytolytic toxins which are often so categorized because colonies of the bacteria which secrete them produce zones of clearing on blood agar media as a result of erythrocyte lysis. Not surprisingly further work on these toxins concentrated on, and made use of, their hemolytic properties.

It is difficult to determine the localized concentrations of toxins in an infected organism, and therefore the toxin concentrations used for *in vitro* experiments may be of little *in vivo* relevance. Certainly in the early stages of an infection the concentration of toxins will be very low. For the enzymatic toxins, low concentrations may still result in a full characterized toxic effect, since it is possible for only a single toxin molecule to be toxic to a target cell. For example, it has been proposed that the entry of a single diptheria toxin enzymatic A subunit into the target cell cytosol is sufficient to kill the cell (Yamaizumi *et al.*, 1978). For the oligomer-forming toxins a minimum threshold toxin concentration must be exceeded to lyse cells, since many subunits must bind to any one target cell to oligomerize and form sufficient functional pores to irreversibly disrupt the cells osmotic balance. Walev *et al.*, (1994) have shown that cells may be able to recover

if only a small number of membrane channels are generated by the toxin. In light of this, it is interesting to note that recently a number of researchers have been investigating the effects of a variety of bacterial protein toxins on cells at lower concentrations than have been used in most earlier work. Several cell types have been used for these experiments but there is a focus of interest upon the cells of the immune system. Since the immune system is involved in the early stages of the host response to bacterial invasion, its cells would be logical targets for the toxins (even at the low concentrations of early infection) to help the pathogen successfully invade the host (Scheffer *et al.*, 1988; König *et al.*, 1994).

Suttorp *et al.* (1985) found that sublytic concentrations of *S. aureus* α-toxin (less than 10 μg/ml) are able to stimulate prostacyclin production in endothelial cells. It has been proposed that this results from the influx of Ca^{2+} through α-toxin pores in the membrane, which initiated an intracellular arachidonic acid cascade. In later work (Bhakdi *et al.*, 1984; Walev *et al.*, 1993; Jonas *et al.*, 1994) these researchers went on to suggest that at low concentrations, α-toxin forms mainly small pores in target cell membranes after binding to high-affinity receptors, but that at higher toxin concentrations a heterologous population of pores is formed as a result of nonspecific interaction with the membrane. These larger pores are required for cell lysis; when α-toxin concentrations are low, the smaller pores only allow the movement of small ions in and out of the cell and stimulate alternative toxic responses. For example, in T-lymphocytes, Jonas *et al.* (1994) reported internucleosomal DNA degradation and apoptosis after exposure to <200 nM α-toxin.

Recently it has been demonstrated that *E. coli* hemolysin can also stimulate immune cells to release inflammatory mediators. Sublytic concentrations of the toxin caused superoxide and leukotriene production in neutrophils, and histamine release from monocytes (Bhakdi and Martin, 1991; Grimminger *et al.*, 1991; König *et al.*, 1994). These responses were dependent upon the formation of transmembrane pores. However, a reduction in cytokine release from monocytes, which was seen after treatment with wild type hemolysin, was reversed when a mutant hemolysin, which was defective in receptor binding but which still formed functional membrane pores, was used instead. This suggested that cytokine suppression was not a result of ion flux through the transmembrane pore but was dependent upon toxin-binding to the cell surface receptor (König *et al.*, 1994).

Although the best characterized role of the thiol-activated toxins is that of forming pores in target cell membranes (typically erythrocytes) there is evidence for alternative cytotoxic mechanisms when different cell types are exposed to the toxins, often at concentrations which are sub-lytic (Boulnois, 1991; Bream *et al.*, 1985; Rubins *et al.* 1994).

Aerolysin is best characterized as a hemolysin; however, enterotoxigenic activity has also been observed (reviewed by Janda, 1991). Asao *et al.* (1984) demonstrated that a preparation of aerolysin purified to homogeneity caused fluid accumulation in mouse intestines and rabbit ileal loops, which are results characteristic of enterotoxins. In two later papers (1986; Kozaki *et al.*, 1988), they demonstrated that preincubation of aerolysin with antiserum directed against the toxin could inhibit these enterotoxic effects.

These results were confirmed by Bloch and Monteil (1989), who concluded that aerolysin could be an important virulence factor in intestinal *A. hydrophila* disease. It is not clear from these results how aerolysin initiates intestinal fluid secretion or even whether pore formation is required.

There is some evidence that bacterial toxins best characterized for specific enzymatic activities can also produce potentially important effects in cells of the immune system. Stewart *et al.* (1989) discovered that pertussis toxin, or the pertussis toxin B-pentamer only, could stimulate mitogenic activity in a T-lymphocyte cell line by increasing inositol phosphate production and Ca^{2+} levels in the target cells. The independence of this response from the presence of pertussis toxin's ADP-ribosyltransferase active A subunit suggests that an interaction with the toxin's membrane receptor is sufficient to stimulate T cell growth.

Hanna *et al.* (1993) have demonstrated that anthrax toxin stimulates macrophages when present at low concentrations. These authors have proposed a mechanism where the low levels of anthrax lethal toxin present in the early stages of an infection stimulate the production of reactive oxygen intermediates and cytokines. As the infection proceeds, toxin levels increase and macrophages are lysed, releasing their contents into the surrounding tissues. It may be this uncontrolled escape of the immune system's own destructive potential that causes the sudden death of the host associated with anthrax (Hanna, *et al.*, in press).

Glycosyl-Phosphatidylinositol Anchored Membrane Proteins

In 1976, Ikezawa *et al.* observed that treatment of slices of rat kidney tissue with the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) resulted in the release of alkaline phosphatase activity. Low and Finean (1977) independently confirmed that PI-PLC could release alkaline phosphatase activity from a variety of mammalian tissues, including lymphocytes. Low and Zilversmit (1980) went on to demonstrate that once released, the alkaline phosphatase could not return to a membrane-bound state. They concluded that the enzyme had been anchored to the membrane via a covalent linkage to phosphatidylinositol. Since then, close to a hundred proteins have been identified which are bound to cell membranes via glycosyl-phosphatidylinositol (GPI) anchors, the chemical structures of the linkages have been elucidated, and many details of the biosynthetic pathway for these proteins have been revealed (reviewed by Ferguson, 1992).

The discovery that the variable surface glycoprotein (VSG) of African trypanosomes is GPI-anchored to the protozoan's membrane provided researchers with a model system to study this form of protein modification (Ferguson *et al.*, 1985; Masterson *et al.*, 1989). Trypanosomes, the causative agents of nagana in cattle and African sleeping sickness in humans, are thought to protect themselves from the host immune system by covering their own plasma membranes with a glycoprotein coat, made up of a million copies of the 60 kDa VSG (Cross, 1975; reviewed by Ferguson 1992). Periodically, the organism is able to switch off production from one VSG gene and begin synthesis from another which is antigenically different, thus avoiding the immune

response which the host may have mounted against antigenic determinants from the old VSG sequence. Since each of these proteins is anchored to the trypanosome membrane via GPI, the organism is the most prolific producer of GPI-anchored proteins known.

Although many of the details relating to the structure and synthesis of GPI anchors have been determined using the trypanosome, work on mammalian proteins has revealed that the system is highly conserved. The anchor consists of a core structure (Figure 2) to which variable glycan side chains are added in different species. One difference between the protozoan and the mammalian GPI anchor core structure is the substitution of the diacylglycerol unit in the former with 1-alkyl,2-acylglycerol in all the mammalian proteins for which the structures have been fully determined (Singh *et al.*, 1994). The core structure is added as a block in the rough endoplasmic reticulum to those nascent polypeptides which bear the appropriate recognition sequences (Vidugiriene and Menon, 1994; Singh *et al.*, 1994).

Proteins predestined to have a GPI anchor have been shown to be transcribed with a characteristic C-terminal signal sequence, in addition to the familiar N-terminal signal sequences which direct them into the endoplasmic reticulum and are removed co-translationally once they have done so. The C-terminal signal has some structural similarities with these N-terminal sequences; both comprise 15 to 30 largely hydrophobic amino acids (Udefriend *et al.*, 1992). By creating chimeras with synthetic C-terminal signal constructs (based on the sequences known to date) attached to a gene for a protein which does not normally become GPI-anchored, it has been possible to determine many optimal characteristics of these sequences. The C-terminal sequence is cleaved off at a

site which consists of three amino acids. The first, which is destined to become the new C-terminus for the protein and to which the GPI anchor is attached via an amide bond to ethanolamine, must be a small residue (alanine, glycine or serine) but not threonine. The amino acid in the second position, immediately after the cleavage point, is less stringently defined (although serine, alanine, glycine, asparagine, aspartic acid or cysteine are preferred) and the third position must be another small residue (Coyne *et al.*, 1993). The cleavage site is separated by about 10 residues from a stretch of at least 11 hydrophobic amino acids at the C-terminus (Coyne *et al.*, 1993), which may be inserted into the endoplasmic reticulum membrane (Low, 1987).

GPI anchors have been found on a wide range of different proteins, including enzymes (such as alkaline phosphatase), cell adhesion molecules (*e.g.* the neuronal cell adhesion molecule, N-CAM), lymphoid antigens (*e.g.* Thy-1), surface coat proteins (*e.g.* variable surface antigen), and cellular receptors (*e.g.* folate receptor) and they are found in an equally diverse variety of organisms and cell lines (reviewed by Cross, 1990; Ferguson, 1992). The absence of any apparent common properties for these proteins, other than their being GPI-anchored, makes it difficult to understand what functional advantage the cell gains by anchoring proteins in this way. Four different hypotheses have been put forward: GPI-anchoring of proteins may help the cell to target the protein to a particular site; enable the selective release of the protein under certain conditions; enhance the motility of the protein in the cell membrane; or enable internalization of the protein by a novel endocytotic mechanism. These hypotheses and some of the evidence for each are outlined below.

Many cells have polarized membranes, and the mechanisms by which some proteins are targeted to one region of the cell membrane and others to a different region remain unclear. In a number of cell types, there is evidence that GPI-anchored proteins are directed to one specific area of the cell membrane. For example, Dotti *et al.* (1991) showed that Thy-1 is transported exclusively to the axonal membranes of hippocampal neurons.

The GPI-anchor can be specifically cleaved by bacterial phospholipase C enzymes, releasing the protein from the cell. It has been demonstrated that *Trypanosoma brucei* has an endogenous phospholipase C activity (Mowat and Clayton, 1987; Lamont, 1989), although there is no evidence to show that this enzyme functions *in vivo* to strip off the VSG coat. A plasma GPI-specific phospholipase D has been identified and characterized but it seems to only be active against detergent solubilized GPI-anchored proteins (Ferguson, 1992; Li *et al.*, 1994).

Metz *et al.* (1994) showed that an endogenous glycosylphosphatidylinositol phospholipase D (GPI-PLD) of HeLa cells can release the complement regulatory protein decay-accelerating factor from these cells. In addition, they showed a corresponding GPI-PLD activity in bone marrow stromal cells, which caused the release of basic fibroblast growth factor-binding heparan sulfate proteoglycan from these cells. They examined cDNA libraries of both cell types, using southern blotting with specific primers, to confirm that the GPI-PLD activity was derived from the cells and not from an external source. This discovery lends weight to the hypothesis that GPI-anchored proteins on the surface of cells may be released from those cells by the action of specific

phospholipases. In addition, phospholipase C cleavage leaves a diacylglycerol in the cell membrane which can act as a second messenger in signal transduction pathways, stimulating a cellular response (Metz *et al.*, 1994; Cross, 1990; Nishizuka, 1984).

GPI-anchoring of proteins may correlate with elevated motility of these proteins compared to conventionally anchored proteins (Noda *et al.*, 1987; Ishihara *et al.*, 1987) although there is evidence that the mobility of proteins in bilayers is determined by factors other than simple hydrodynamic size, such as interaction with other membrane components (Phelps *et al.*, 1988). Proteins can be attached to cell membranes via a number of mechanisms (reviewed by Jennings, 1989). The most conventional type of membrane protein anchor is a membrane spanning domain formed by part of the protein's own sequence. For some proteins (*e.g.* porins or other membrane pores or transporters), much of the protein is buried in the membrane as a series of transmembrane spans with a number of short loops protruding on both the cytoplasmic and extracellular membrane faces. Other proteins have a single transmembrane span (*e.g.* glycophorin), perhaps as short as 15 amino acids, with a larger domain on either side. A third group of proteins has very short hydrophobic sequences which insert into the membrane anchoring the protein but none of the amino acid sequence emerges on the other side of the bilayer (*e.g.* cytochrome b5). The last group could exhibit membrane motilities equal to those of GPI-anchored proteins since they do not have large integral membrane domains or cytoplasmic domains to move, but this method of membrane attachment is extremely rare.

A novel mechanism for the uptake of extracellular ligands may involve binding to a GPI-anchored receptor and internalization via non-coated pits. The classical pathway for receptor-mediated endocytosis begins with the binding of ligand to the receptor (reviewed by Trowbridge *et al.*, 1993). Receptor-ligand complexes cluster in clathrin-coated pits and are eventually endocytosed. The receptor may be recycled back to the membrane from the endosome, while the ligand, and any receptors which are not recycled, are transferred to a cellular compartment (*e.g.* a lysosome) for processing. Rothenberg *et al.* (1990) discovered that the folate receptor, a GPI-anchored protein, is localized on the cell membrane in uncoated invaginations, but can bind and effect the internalization of 5-methyltetrahydrofolic acid without ever being associated with lysosomes or endosomes. These results suggest that this receptor employs a novel mechanism to deliver its ligand to the cell cytoplasm that may be possible because of the folate receptor's GPI-anchor.

It would appear that there may be no single reason why any given protein is GPI-anchored. Future work may uncover more uses for GPI-anchoring of proteins by cells and may show that one of the above hypotheses is the explanation in the majority of instances where proteins are GPI-anchored.

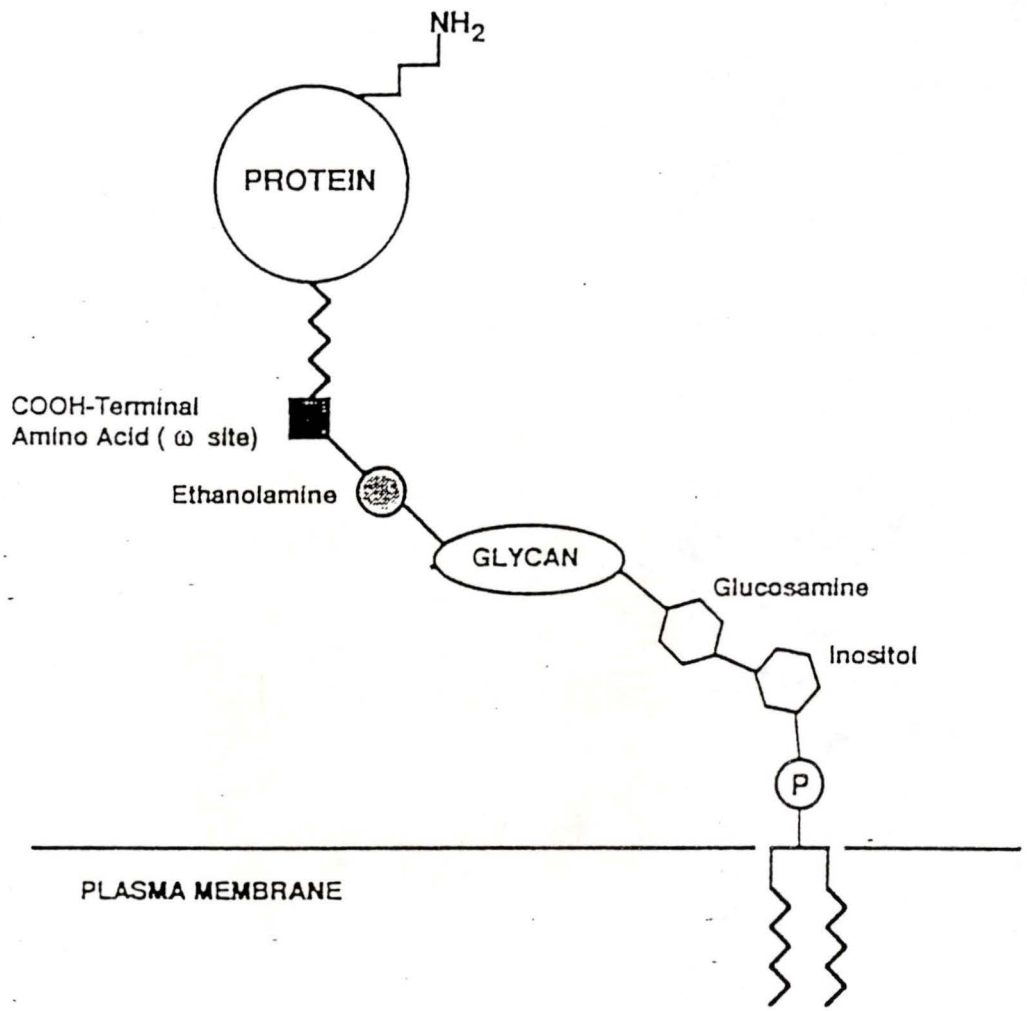

Figure 2: The GPI-anchor common core structure. (Adapted from Udenfriend *et al.*, 1992)

The ADP-Ribosylation Cycle

As has been described above, a number of bacterial protein toxins function by catalyzing the ADP-ribosylation of target proteins in eucaryotic cells, causing either inactivation (*e.g.* DT on EF2) or activation (*e.g.* CT on $G_{s\alpha}$). The consequences of the actions of these toxins first demonstrated the potency of ADP-ribosylation as a potential means of regulating protein activity. The discovery of a number of eukaryotic ADP-ribosyltransferases (Okazaki *et al.*, 1994; Donnelly *et al.*, 1994), and their opposing ribosylhydrolases (Takada, *et al.*, 1993; Moss *et al.*, 1992; 1993) has revealed that regulatory cycles involving ADP-ribosylation exist within eucaryotic organisms.

It is becoming clear that ADP-ribosylation is both a potent and a widespread mechanism for regulation of protein activity, and hence numerous cellular processes. Future research will doubtless reveal many more enzymes which are involved in the ADP-ribosylation cycle, and through the precise identification of their target proteins help us to understand the cellular roles of this new family of eukaryotic enzymes.

ADP-ribosyltransferases (ADP-RTs) can be grouped according to biochemical activity. Mono-ADP-RTs add a single ADP-ribose group at each amino acid residue modified via an N-glycosidic linkage. To date, mono-ADP-RTs have been identified which modify arginine (*e.g.* cholera toxin, or rabbit skeletal muscle ADP-RT), cysteine (*e.g.* pertussis toxin or human erythrocyte ADP-RT), diphthamide (*e.g.* diptheria toxin, or bovine hepatic ADP-RT), asparagine (*e.g.* botulinum C3 toxin) and lysine (reviewed by Donnelly *et al.*, 1994). The bacterial mono-ADP-RTs appear to be structurally unrelated to their eukaryotic equivalents and do not exhibit much sequence similarity to them,

although there are three small regions found in all the ADP-ribosyltransferases which share some homology (Takada, *et al.*, 1995). These regions are therefore likely to be functionally important and the result of convergent evolution.

Poly-ADP-RT activity results in the attachment of chains (sometimes branched) of O-linked ADP-ribose subunits to proteins at a specific amino acid residue (Banasik *et al.*, 1992). The enzyme is able to catalyze both the initial transfer of ADP-ribose from NAD^+ to the protein (glutamate is the most common acceptor) and the subsequent polymerization of ADP-ribose units onto the growing chain. Most of the poly-ADP-RTs which have been identified are nuclear enzymes and numerous nuclear proteins, including histones, have been identified as targets (reviewed by Ueda and Hayashi, 1985).

Recently, a mono-ADP-ribosyltransferase was discovered that has a GPI-anchor, and is attached to the cell's plasma membrane, apparently facing the extracellular environment. Zolkiewska *et al.* (1992) cloned and characterized this arginine-specific mono-ADP-ribosyltransferase from rabbit skeletal muscle sarcolemma. The protein contains a hydrophobic C-terminal signal sequence which has the characteristics of a GPI attachment site. They demonstrated that this enzyme was GPI-anchored by showing that a membrane fraction from cells transformed with a vector encoding the ADP-ribosyltransferase gene could transfer ADP from NAD^+ to agmatine and that this ADP-ribosylase activity was released by PI-PLC treatment of the cells (Okazaki *et al.*, 1994). The protein was found to be homologous with the RT-6 surface antigens of rat and mouse post-thymic T-cells which are GPI-anchored and have subsequently been shown to

possess NAD^+-glycohydrolase activity (Takada *et al.*, 1994). More recently, several other GPI-anchored NAD^+:arginine ADP-RTs have been identified in chicken bone marrow cDNA libraries (Tsuchiya *et al.*, 1994), and human skeletal muscle cells (Okazaki *et al.*, 1994; Klebl *et al.*, 1994), all of which show a high degree of sequence conservation with each other and with the RT-6 proteins, but not with the ADP-ribosylating toxins (Okazaki *et al.*, 1994; Tsuchiya *et al.*, 1994).

There is evidence that the muscle cell enzyme can ADP-ribosylate integrin $\alpha 7$ (Zolikiewska and Moss, 1993). The authors propose that the ADP-ribosylation could represent some form of regulation of integrin's receptor function which in turn could determine the way that the muscle cell responds to extracellular stimuli, via signal transduction. A number of GPI-anchored proteins have been identified on the surface of T-cells, including Thy-1, Qa-2, TAP, RT-6.1 and RT6.2. The latter two have been identified as ADP-glycohydrolases and the first three have been shown to associate with non-receptor tyrosine kinases in the plasma membrane (Stefanova *et al.*, 1991; Thomas and Samelson, 1992; Shenoy-Scaria *et al.*, 1992). All of these proteins are thought to have a role in signal transduction, but more work is required to determine what it is.

Pu *et al.* (1995) have recently demonstrated that $HgCl_2$ treatment of T-lymphocytes produces cross-linking of GPI-anchored proteins on the plasma membrane of the cells as well as heavy tyrosine phosphorylation of cellular proteins. If the cells are pretreated with PI-PLC to remove GPI-anchored proteins prior to $HgCl_2$ treatment, there is a corresponding drop in the resultant level of cellular protein phosphorylation. In addition, a cell line defective in one of the genes required for GPI-anchor biosynthesis is

insensitive to $HgCl_2$ treatment, but the signalling pathway can be restored by transfecting these cells with the functional gene. This indicates that the GPI-anchored proteins on these cells can activate a signal transduction pathway in response to stimuli.

Evidence from Wang *et al.* (1994) suggests that GPI-anchored ADP-ribosyltransferases may have an important role in the control of proliferation of cytotoxic T-lymphocytes, CTL. They demonstrated that proliferation of CTL was suppressed after incubation with NAD^+ as a result of the inability of the cells to conjugate with targets and a lack of responsiveness to T-cell receptor mediated stimulation. Using radiolabeled NAD^+, they were able to demonstrate that cellular proteins were ADP-ribosylated on arginine residues as part of the response. Pretreatment of the cells with PI-PLC released an NAD^+:arginine ADP-ribosyltransferase activity and reduced the sensitivity of the CTL to NAD^+.

MATERIALS

Media and Reagents

Indolylphenylthiogalactoside (IPTG) and phosphatidylinositol-specific phospholipase C (PI-PLC) were purchased from Boehringer Mannheim. 1,10-phenanthroline was supplied by Aldrich chemicals. Wheat germ agglutinin, concanavalin A, Sodium N-laurylsarcosine (SLS), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and t-octylphenoxypolyethoxyetanol (Triton-X-100) were purchased from Sigma. Chromatography matrices [DEAE-sepharose CL-6B, CM-sepharose CL-6B, wheat germ lectin sepharose 6MB (WGA-sepharose), CNBr activated sepharose, activated thiol-sepharose 4B and prepacked PD-10 columns], were from Pharmacia. Solvents, both reagent grade and HPLC grade (used for N-terminal sequencing gels) were supplied by Anachemia. Octyl polyoxyethylene (Octyl Pol) was from Bachem Bioscience Inc. Acrylamide and N,N'-methylene-bisacrylamide for polyacrylamide gel electrophoresis were from BDH. NaCl, ammonium sulfate for aerolysin precipitation, tris(hydroxymethyl)aminomethane (Tris), NaOH, $MgCl_2$, $CaCl_2$, ethylenediaminetetraacetic acid (EDTA), dimethyl formamide (DMF) and glycerol were from BDH. Skim milk powder was from Oxoid. Sodium dodecyl sulfate (SDS) and polyoxyethylene-20-sorbitan monolaurate (Tween-20) were from Fisher Scientific. $Na^{125}I$, ^{35}S-methionine (^{35}S-Met) and reagents for enhanced chemiluminesence were supplied by Amersham. All other reagents were supplied by Sigma unless otherwise specified.

Luria-Bertani (LB, supplied by Difco) media was prepared as described by Sambrook *et al.* (1989) except that the pH was adjusted to 7.5. One part 10 x modified Davis buffer (Ashton *et al.*, 1980) was added to nine parts LB to prepare LB-Davis media. Glucose was added 1:100 to media when required from a 20% sterile stock solution. Riddle's media (Riddle *et al.*, 1981) contained 0.1% histidine and 0.1% glutamic acid, but not cobalt chloride, copper sulfate or magnesium chloride. Phosphate buffered saline (PBS) was 10 mM NaH_2PO_4 pH 7.4, 0.15 M NaCl. PBS-T was made by the addition of 0.5% Tween-20 to PBS. 4 X sample buffer for SDS-PAGE samples (8% SDS, 20% β-mercaptoethanol, 40% glycerol, 108 mM H_2SO_4, 220 mM Tris-HCl pH 6.1 with bromophenol blue) was added 1:3 to liquid samples, which were sometimes diluted in dH_2O. 1 X sample buffer, for resuspending pellets or dried samples, was prepared by diluting 4 X sample buffer 1:3 with dH_2O. For determination of radioactivity in samples using a liquid scintillation counter, 1 ml Beckman Ready Safe scintillant was added to each sample.

Equipment

Optical density was measured using a Gilford Response UV-VIS spectrophotometer. ^{35}S-Methionine was measured using a Beckman LS 6000 TA scintillation counter. ^{125}I was counted on this counter for β-emissions or on an LKB 1282 COMPUGAMMA gamma counter. *A. salmonicida* was grown up as a 2 liter culture in a Virtis fermenter, or as three 650 ml cultures in 2 liter flasks in a New Brunswick Scientific controlled

environment incubator shaker. Columns, column accessories, pumps (P-3 or P-1) and fraction collectors were all made by Pharmacia.

Bacterial Strains and Plasmids

Aerolysin cysteine mutants were constructed by Angela Schulze by site directed mutagenesis and cloned into pMMB66HE for expression in *A. salmonicida* CB3, a protease-deficient mutant of rifampicin resistant *A. salmonicida* 440 (see Table 1), obtained by Tn5 mutagenesis (Buckley 1990). Cys 159 to Ser has a free cysteine at position 164, and Ile 445 to Cys, Gly 202 to Cys both have an extra free cysteine thiol group compared to wild type aerolysin.

Wild-type (WT) and site directed mutant proaerolysins were expressed in *A. salmonicida* strain CB3 (Buckley, 1990). For the preparation of ^{35}S-methionine labeled proaerolysin, a pirazmonam resistant strain of *A. hydrophila*, 2H^{-} was used (Wong, 1994), which also is protease-deficient in early to mid-log phase. In contrast to *A. salmonicida*, *A. hydrophila* strains can be grown in Riddle's media. Use of a defined media allowed us to control the amino acid composition of the growth media, and to supplement it with ^{35}S-methionine.

Name	Description	Reference
Strains		
CB3	Protease deficient *A. salmonicida* 440 strain prepared by Tn5 mutagenesis.	Buckley, 1990
2H-	Protease deficient pirazmonam resisitant *A. hydrophila* AH65 strain	Wong, 1994
Plasmids		
pKWB5.3	pMMB66HE Ω (pPH501, NsiI-BclI, 1.7 kb) Cm^r	Wong, 1994
pMMB66HE	RSF1010 Δ (Psti-PvuII, 2.8 kb) Ω (lacI tacP rrnB bla NruI-AhaIII, 3.0 kb) Ap^r	Furste *et al.*, 1986

Table 1: Strains and plasmids used for the production of aerolysin in *Aeromonas* species.

METHODS

General Laboratory Protocols

Bacterial Culture

All *Aeromonas* cultures were grown at 28°C and 250 rpm, either in a New Brunswick Scientific Controlled Environment (model G-25) shaking air incubator or a Virtis fermenter. Unless stated otherwise, cultures were grown to optical density at 600 nm of 0.5 before isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM final concentration.

Preparation of Washed Packed Erythrocytes from Whole Blood

One volume whole blood (outdated human blood was collected from the Jubilee Hospital blood bank, heparinised animal blood was obtained fresh from the animal care facility) was diluted with three volumes PBS. The suspension was centrifuged for 5 minutes at 2,000 x g and the supernatant was removed using a tap aspirator. The buffy coat was carefully aspirated. The blood was resuspended in the same volume of PBS, centrifuged and aspirated as before. This cycle was repeated until the supernatant was clear (typically 3 to 4 centrifugations in all).

Purification of Proaerolysin

An overnight culture of *A. salmonicida* strain CB-3 was used to inoculate 2 liters of LB media 1:200 (in the fermenter vessel, or divided equally between three 2 liter flasks

for growth in the air incubator) and the culture was grown to OD_{600} of 0.5. It was then induced to express aerolysin by adding IPTG to 1 mM final concentration, and grown for a further 12 to 19 hours at 30°C. The supernatant was collected by pelleting the cells for 20 minutes at 10,000 rpm at 4°C in a JA-14 rotor (Beckman). Phenanthroline (a Zn^{2+} protease inhibitor; 10 μM final concentration) was added to the supernatant to inhibit proteolysis. The supernatant proteins were concentrated from 2 liters to approximately 50 ml, either by ammonium sulfate precipitation (60% saturation at 0°C) or using a concentrator. The concentrate was centrifuged at 39,000 rpm at 4°C for 2 hours in an SW-41 rotor (Beckman), and the supernatant collected and desalted on a G-25 column. Protein-containing fractions (as identified by OD_{280} measurement) were pooled and applied to a hydroxy-apatite column, then eluted with a 20 to 150 mM phosphate gradient in 0.3 M NaCl. The aerolysin peak was identified by measuring OD_{280} of the eluted fractions and using the aerolysin titre protocol given below to determine the hemolytic activity of the peak fractions. The hemolytic peak fractions were pooled and protein was precipitated by ammonium sulfate (60% saturation at 0°C) and resuspended in 20 mM HEPES pH 7.4 for applied to a DEAE-sepharose column. Aerolysin was eluted using a 0.1 M to 0.5 M NaCl gradient in 20 mM HEPES pH 7.4, and the peak fractions, monitored at 280nm, were stored at -70°C.

Hemolytic Titres

A 96-well microtitre plate was set up as follows: typically, 1-10 μg of proaerolysin, or 10 to 50 μl of unknown sample, were placed in the first well of the

microtitre plate. Proaerolysin was activated by the addition of 0.2 μg of trypsin and the total volume was made up to 100 μl with PBS. The trypsin activation proceeded for 10 minutes at room temperature. Meanwhile 100 μl of PBS were added to the remaining 11 wells in each row on the plate. After activation, the aerolysin was serially diluted (1:2) along each lane (for 12 to 15 wells). One hundred microliters of 0.8% washed and packed RBCs (prepared as described below) were then added to each well. The number of wells showing partial or complete clearing over incubation time was observed. The addition of 10 μg aerolysin to the first well would clear 10 wells of 0.4% human RBCs after 60 minutes incubation at 37°C. This would be recorded as a titre of 10.

For some titres, where the ability of a solution or a suspension to inhibit aerolysin hemolysis was being investigated (eg. the PI-PLC supernatants and glycophorin preparations), an additional preincubation step was included to allow time for activated aerolysin to combine with potential receptor prior to the addition of the erythrocytes. The proaerolysin was activated as above with trypsin for 10 minutes at room temperature. The trypsin was then inactivated with a five-fold molar excess of soybean trypsin inhibitor before the test sample or control solution was added. The mixtures were then preincubated at room temperature or 37°C before adding RBCs.

In the experiments to determine the inhibitory capacity of PI-PLC erythrocyte digest supernatant, 10 μg proaerolysin was activated in a total volume of 100 μl in the first well as described. Fifty microliters PBS was added to all the other wells along each row, and after the addition of a tenfold molar excess of trypsin inhibitor, 50 μl from the first well was serially diluted 1:2 along each lane. Fifty microliters of the test supernatant

was added to each well, starting with the wells containing the lowest concentration of aerolysin to be tested [i.e. from the 15th well for rat RBC titres (there was insufficient supernatant volume for additions to be made to the first two wells of these lanes, so in these two wells the volume was made up with PBS), and from the 11th for human RBC titres]. The plates were preincubated for 10 minutes at room temperature before the addition of 100 µl 0.8% RBCs to each well.

For titres using *Staphylococcus aureus* α-toxin, rabbit erythrocytes were prepared from fresh heparinised rabbit blood as described for rat erythrocytes and treated in the same way with PI-PLC (as described below) before being titred with two hemolytic units of α-toxin (Calbiochem).

Determination of Protein Concentration

Aerolysin concentrations were determined by measuring OD_{280}, taking a value of 2.2 to represent a protein concentration of 1 mg/ml (Buckley, unpublished results). In some samples, protein concentration could not be measured by measuring OD_{280} (for example in erythrocyte ghosts). In these cases, protein concentrations were determined using the BIO-RAD DC protein assay kit according to the manufacturers instructions.

Gel Electrophoresis Techniques

Sodium-Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS polyacrylamide gels were prepared as described by Neville (1971) using the BIO-RAD mini-protean slab gel casting system. A 12% separating gel and a 3% stacking gel were used unless otherwise stated. Samples were prepared in protein sample buffer and boiled for three minutes before being loaded onto the gel. Gels were run at 16 mA (maximum 60 V) constant current until the dye front reached the separating gel, then at 40 mA (maximum 160 V) constant current until the dye front reached the bottom of the gel. Proteins were either stained by one of the methods given below, or transferred to nitrocellulose, as described.

Visualizing Proteins in Polyacrylamide Gels

i) Staining with Coomassie brilliant blue R-250

Gels were fixed and stained in 0.03% Coomassie blue, 25% isopropanol, 10% acetic acid for 15 minutes or until bands were visible, then gradually destained by transferring to solutions with progressively lower Coomassie blue concentrations. In preparation for drying, gels were destained fully by washing for 1 to 2 hours in 10% acetic acid, then soaked in water for 10 minutes before being transferred to Watman No. 3 paper and dried under vacuum at 80^{o}C.

ii) Silver staining

Gels were fixed in 50% methanol, 10% acetic acid for a minimum of 30 minutes, then rehydrated in 10% methanol, 10% acetic acid with heating for 1 minute in a microwave, followed by a 5 minute wash in water. The water was drained and 33.3 μM dithiothreitol was poured over the gels, and this was heated for 1 minute in a microwave. The gels were rinsed with water and incubated in 0.1% (w/v) $AgNO_3$ for 15 minutes. Excess $AgNO_3$ was washed away thoroughly with dH_2O before the gels were developed in 3% Na_2CO_3 (w/v), 0.037% formaldehyde (v/v). Once the protein bands were clearly visible, development was stopped with 5% acetic acid.

iii) Periodic acid / Schiff's staining

Gels were fixed in 25% isopropanol, 10% acetic acid for at least 30 minutes, then incubated on a shaker at room temperature as follows:

20 minutes in 5% isopropanol, 10% acetic acid; 60 minutes in 0.5% periodic acid; 30 minutes in 0.5% sodium arsenite solution in 5% acetic acid; two times, for 20 minutes each in 0.1% sodium arsenite in 0.5% acetic acid; and finally, briefly in 5% acetic acid. Glycoprotein bands were developed by incubation for 2 hours or more in Schiff's reagent (basic fuchsin in 0.1M HCl) then gels were destained in several changes of 0.6% (w/v) sodium metabisulphite in 0.01 M hydrochloric acid. Once destained, the gels were dried as described previously.

To improve the staining of rat glycoproteins which are O-acetylated (Sarris and Palade, 1979), a 0.1 N sodium hydroxide wash was included immediately after fixing,

followed by extensive washing in the fixing buffer, before proceeding with the series of incubations outlined above.

Preparing N-terminal Sequencing Gels

All glassware for this procedure was washed with 90% HNO_3, 10% HF then rinsed thoroughly with distilled water before use. Plastic ware, including gel running and blotting apparatus, was soaked overnight in Decon solution (prepared according to manufacturers instructions) then rinsed thoroughly with distilled water. Gel solutions were prepared fresh, using HPLC grade dH_2O. A 12% SDS-polyacrylamide gel was poured according to Laemmli (1970) and allowed to polymerize overnight at room temperature before use. A 3% stacking gel was poured and allowed to polymerize for at least two hours before the gel was prerun at 5 mA for 2 hours with 0.05 mM glutathione added to the upper running buffer to remove any free radicals or oxidizing groups. Thioglycolate (0.1 mM) was included in the upper reservoir buffer to quench reactive groups which might lead to N-terminal blocking of the proteins. Protein samples were boiled in 1 x sample buffer, then loaded into the wells. Samples for sequencing were loaded into the center lanes, flanked by molecular weight standards, and additional samples were loaded into the lanes outside the markers. After blotting, these outside lanes were cut off and developed using the alkaline phosphatase method described to reveal the exact position of the 47 kDa blot band. By repositioning these lanes flanking the blot, it was possible to cut out the receptor band from the center lanes of the blot for N-terminal sequencing. The gel was run at 5mA until the dye front reached the bottom of

the stacking gel (3-4 hours). The gel was then soaked in transfer buffer (0.1 M CAPS pH 11.0, 10% MeOH) before prepared PVDF membrane was applied and the proteins transferred as described under Western blotting. After blotting, the membrane was washed three times with dH_2O before being dried, ready to be applied to the N-terminal sequencer.

Western Blotting

Proteins were transferred from polyacrylamide gels to nitrocellulose (BIO-RAD) or poly-vinylidene diflouride (PVDF) membrane (Applied Biosystems Problott membrane) in a BIO-RAD mini-cell transfer apparatus (wet cell) for 60 minutes at 90 V as described by Towbin *et al.* (1979).

Visualization of Proteins Immobilized on Membranes

i) Alkaline phosphatase (the aerolysin sandwich Western blot method)

Membranes were blocked with 5% (w/v) skim milk in PBS-T for at least 45 minutes at room temperature, or overnight at 4°C. All subsequent incubations were performed at room temperature on a lab shaker, unless otherwise noted. The aerolysin receptor band was visualized as follows: the membrane was incubated with proaerolysin, or aerolysin (1 μg/ml) in PBS-T for 45 minutes; washed three times for 5 minutes each with PBS-T and then incubated with mouse monoclonal anti-aerolysin ascites (1:4000 in PBS-T) for 60-90 minutes on a lab shaker. The membrane was again washed three times for 5 minutes with PBS-T and then incubated with goat anti-mouse IgG (H&L) alkaline

phosphatase conjugate (from Caltag; 1:4000 in PBS-T) for 60 minutes. After this the blot was washed three times for 5 minutes with PBS-T, then washed for five minutes in 100 mM NaCl, 50 mM Tris-HCl pH 9.5, 100 mM $MgCl_2$ and developed with nitro-blue tetrazolium (NBT; 0.330 mg/ml), bromochloroindolylphenol (BCIP; 0.167 mg/ml) in this buffer for between 5 minutes and 2 hours.

ii) Enhanced chemiluminescence (ECL)

To increase sensitivity, some blots were developed using the Amersham ECL system. Western blotting was performed as described, and the procedure followed was as for alkaline phosphatase with the following exceptions: Caltag second antibody was omitted, and in its place a 1:4000 solution of Amersham goat anti-mouse IgG Horse-Radish Peroxidase conjugate was used. After 1 hour of incubation with this antibody conjugate the blots were washed eight times in PBS-T, three times for 5 minutes, twice for 15 minutes then a further three times for 5 minutes, before being treated with ECL reagent and exposed to film according to the manufacturer's instructions.

iii) Biotinylation of glycoproteins

Rat erythrocyte membranes were prepared according to method 1 as described. 10 µl of a 10 mg/ml protein rat erythrocyte membrane suspension (in 20 mM NaH_2PO_4, pH 7.4) were added to 90 µl acetate buffer (100 mM NaOAc pH 5.5), pelleted, aspirated and washed twice more with 100 µl acetate buffer, then resuspended in 25 µl acetate buffer. A 20 µl aliquot of this suspension was mixed with 10 µl of 30 mM $NaIO_4$ and incubated for 30 minutes in the dark at room temperature. Membranes were pelleted,

aspirated and washed twice with 30 µl acetate buffer, then resuspended in 20 µl acetate buffer, to which 10 µl of 5 mM biotin LC-hydrazide (PIERCE; prepared in acetate buffer) was added. This mixture was incubated for 1 hour at room temperature in the dark before the reaction was stopped by the addition of 50 µl of 100 mM Tris-HCl (pH 7.5). The membranes were pelleted and washed again twice with 100 µl acetate buffer before being resuspended in 20 µl 1 X sample buffer and loaded onto a SDS-polyacrylamide gel and blotted. After blocking overnight with 3% BSA, the blot was probed with 1:3000 streptavidin-alkaline phosphatase conjugate, washed, and then developed with NBT / BCIP as described above.

iv) Probing blots by using biotinylated lectins

NHS-Biotin (2 µl of 50 mg/ml in dimethylsulfoxide; PIERCE) was added to 1 mg lectin (concanavlin A, or wheat germ agglutinin) in 0.5 ml PBS, and incubated for 30 minutes at room temperature before being dialyzed for 24 hours against PBS.

The biotinylated lectins were used to visualize glycoproteins immobilized on membranes as described above (alkaline phosphatase) with the following modifications: blots were blocked overnight using 3% periodate treated BSA. Biotinylated lectins were substituted in place of the first antibody, mouse anti-aerolysin monoclonal in the original alkaline phosphatase method. Second antibody (goat anti-mouse IgG (H&L) alkaline phosphatase conjugate in the alkaline phosphatase method) was substituted with a 1:3000 dilution of streptavidin-alkaline phosphatase conjugate. No aerolysin or proaerolysin incubation was performed for these blots.

Purifying the Aerolysin Receptor from Erythrocytes

Erythrocyte Membrane Preparation

Erythrocyte membranes were prepared by one of two methods as specified in each case; following either Dodge *et al.* (1962), or Steck and Kant (1974).

Method 1 (Adapted from Dodge *et al.,* 1962).

Washed human or rat erythrocyte cells were lysed by resuspension in 20 volumes of 20 mM NaH_2PO_4 pH 7.4. The membranes were pelleted at 12,000 rpm for 15 minutes at 4°C in a Beckman JA-14 rotor. The pellets were washed in the same volume of this buffer and centrifuged again, and the washing cycle repeated until the supernatants were clear and nearly colourless. The pellets were resuspended to the original volume of packed cells with 20 mM NaH_2PO_4 pH 7.4, and stored at -20°C.

Method 2 (Adapted from Steck and Kant, 1974).

Human, rat or mouse packed red blood cells were diluted rapidly 1:20 in cold lysis buffer [5 mM phosphate pH 8.0, usually containing 0.5mM phenylmethylsufonyl-fluoride (PMSF) and 1 μM pepstatin A]. Membranes were pelleted for 10 minutes at 12,000 rpm, 4°C in a JA-14 rotor (Beckman) after which the supernatants were removed with a tap aspirator, and the dark, solid spots of proteins and white blood cells at the base of the tube were removed. The pellets were resuspended in the same volume of lysis buffer, centrifuged and drained again, and the cycle continued until the membranes appeared to be pale "ghosts". The loose membrane pellets were stored at -70°C.

The protein concentration of the erythrocyte ghosts prepared by either method was found to be between 5 and 8 mg/ml by the BIO-RAD DC assay method.

Detergent Extraction of Proteins from Erythrocyte Membranes

A number of detergents were tested for their ability to solubilize the receptor from rat RBCM. These included CHAPS, Triton-X-100, Tween-20, octyl glucoside, Octyl Pol, and SLS were all tested, both in 0.34 M NaCl and in dH_2O.

Purification of the Aerolysin Receptor from Rat Erythrocytes

Three volumes of rat erythrocyte ghosts (prepared by method 2) were added to one volume of 4 x solubilization buffer (40 mM HEPES pH 8.0, 2% Triton X-100, 2 mM PMSF, 4 μM pepstatin A) and mixed end over end at 4°C for 30 minutes. The membrane debris was pelleted by centrifugation for 25 minutes at 16,000 rpm in a Beckman JA-17 rotor at 4°C. The supernatant was loaded onto a pre-equilibrated DEAE-sepharose (Pharmacia) column washed with 10 to 20 ml of 1 x solubilization buffer. The bound protein was eluted with a 0 to 0.25 M NaCl gradient (in 1 x solubilization buffer). Fractions were tested for the presence of an aerolysin binding protein after separation by SDS-PAGE, using the sandwich Western blot detection method described. The receptor-containing fractions were pooled and loaded onto a WGA-sepharose 6MB (Pharmacia) column with a flow rate of 10 ml / hour. Unbound protein was washed through the column with 10 ml of 1 x solubilization buffer and then the bound protein was eluted

with 0.1 M N-acetyl glucosamine (GluNAc) in the same buffer. The receptor-containing fractions were again detected using the sandwich Western blotting technique.

Acetone Precipitation of Proteins

In some cases, the receptor was acetone-precipitated from the WGA peak fractions to facilitate concentration. Three volumes of acetone (-20°C) were added to one volume column eluate and this mixture was incubated for 30 minutes at -20°C. The precipitate was pelleted at 8,000 rpm for 25 minutes at 0°C in a Beckman JA-17 rotor. The pellet was drained and washed with the same volume -20°C acetone, pelleted (8,000 rpm, 15 minutes, 0°C), drained, and finally washed with the same volume of -20°C ether, pelleted, drained, and dried under vacuum.

Purification of Glycophorin from Erythrocyte Membranes

Glycophorin was prepared from both human and rat erythrocytes following the method of Marchesi and Andrews (1971). Erythrocyte ghosts were prepared as described by method 1 and lyophilized for 30 hours. The yield from 240 ml of human blood was 1.6 g of membrane material, and from 25 ml of rat blood (from a single large male) was 72 mg membranes. Glycophorins were extracted from the membranes using lithium diiodosalicylate (LIS). One milliliter of 0.3 M LIS in 50 mM Tris pH 7.4 was added per 25 mg membranes and the mixture was stirred in darkness at room temperature for 15 minutes. Two volumes of cold dH_2O were added and the mixture was stirred for a

further 10 minutes on ice, in the dark. Membrane debris was pelleted by centrifugation for 90 minutes at 45,000 g at 4°C. An equal volume of 50% (w/w) phenol in water was added to the supernatant and this mixture was stirred for 15 minutes on ice before the phases were separated by centrifugation at 4,000 g for 60 minutes at 4°C. The aqueous phase was collected and dialyzed for 36 hours against dH_2O. The dialysate was lyophilized and the pellet was ethanol extracted twice. This pellet was dissolved in a small volume of water and dialyzed again against dH_2O before finally being lyophilized and resuspended in 20 mM Tris pH 7.4.

Immobilization of Proaerolysin

By immobilizing proaerolysin on Sepharose matrices it should be possible to construct an affinity column for the 47 kDa aerolysin receptor protein to aid in the purification of this protein from rat erythrocyte membranes.

i) Activated thiol sepharose 4B

Site-directed mutants of proaerolysin, each with a single free cysteine group, were immobilized on activated thiol sepharose 4B (Pharmacia) by the formation of a disulfide bond. For each variant, 0.5 to 1.0 mg of proaerolysin (in 3 ml of 20 mM HEPES, pH 7.4) was combined with 0.1 g activated thiol-sepharose resin, which had been prepared according to the manufacturers instructions and equilibrated in HEPES-buffered saline (20 mM HEPES pH 7.4, 0.15 M NaCl; HBS). The protein-resin mixture was incubated for 2 hours at room temperature with end over end mixing, then the resin was pelleted. The OD_{280} of the resultant supernatant was measured to determine how much of the

protein had bound to the resin. The resin was washed twice with 10 ml of HBS, resuspended in 3 ml HBS and stored at 4°C. Aliquots were taken for subsequent experiments.

ii) CNBr activated sepharose 4B

CNBr activated sepharose 4B (0.725 mg; Pharmacia) was hydrated with 5 ml of 1 mM HCl for 10 minutes at room temperature then poured into a small column. The column was washed through with a total of 100 ml of 1 mM HCl and drained; a pump was used to increase the flow rate to 100 ml / hour for this step. 25 mg of wild-type proaerolysin in 3 ml coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl) were run into the column, the ends were sealed, and this mixture was incubated on the shaker for 30 minutes at room temperature under N_2. The gel was drained and washed through twice with 10 ml coupling buffer. Any remaining free protein binding sites were blocked by adding 10 ml of 0.1 M Tris, pH 8.0 and incubating on the shaker for a further 30 minutes. Finally the column was washed three times with 10 ml of 0.1 M acetate buffer pH 4.0, 0.5 M NaCl, followed by 10 ml 0.1 M Tris pH 8.0, 0.5 M NaCl. The column was stored at 4°C in 5 mM HEPES pH 7.4, 0.25 M NaCl , 1 mM $CaCl_2$ containing 5 mM NaN_3.

Characterization of the Erythrocyte Membrane Aerolysin Receptor

De-N-Glycosylation of Glycoproteins

Fifteen microliters of rat erythrocyte ghosts (prepared by method 2; 6.5 mg/ml total protein) were added to an equal volume of 10 mM EDTA pH 8.0, 1% SDS, 10% β-mercaptoethanol and boiled for 2 minutes. The mixture was allowed to cool to room

temperature, then 7.5 µl of 10% octyl glucoside and 22.5 µl peptide-N-glycosidase F (PNGaseF; Oxford Glycosystems) were added sequentially, and the mixture was incubated for 22 hours at 25°C. A control sample was prepared to which 22.5 µl enzyme-free buffer was added in place of the PNGaseF. Aliquots of these samples were run on SDS-PAGE for staining and sandwich Western blotting as described.

Serine Protease Treatment of Red Blood Cells

Either rat or human blood was washed three times in Tris-buffered saline (TBS; 20 mM Tris pH 7.4, 0.15 M NaCl). Trypsin or chymotrypsin (50 µl of 10 mg/ml) were added to 300 µl packed red cells, the volume was made up to 900 µl with TBS and the mixture was incubated for 1 hour at 37°C. The cells were washed three times with 1% BSA in TBS before samples were taken for aerolysin titres. RBCM were prepared according to method 2, dissolved in sample buffer and run on SDS-PAGE for staining and sandwich Western blotting. Control samples were prepared as per the trypsin tube except that a tenfold excess of soybean trypsin inhibitor was added prior to the trypsin.

Proteinase K Digestion of Rat Erythrocyte Ghost Protein

Rat RBCM (75 µl; prepared using method 2) were mixed with 25 µl of 4 x sample buffer and boiled for 5 minutes. The mixture was cooled on ice, then 100 µl proteinase K (1 mg/ml in 20 mM Tris pH 7.4) was added and the mixture incubated for 45 minutes at 60°C. A control sample was prepared in which 20 mM Tris-HCl pH 7.4 was added in

place of the proteinase K solution. Samples were loaded onto SDS-PAGE for staining and sandwich Western blotting as described above.

Aerolysin Inhibition by Human and Rat Glycophorins and Protease Treatment of Purified Human Glycophorin

Human glycophorin was purified and used in aerolysin inhibition titers as described. Different amounts of rat and human glycophorin were preincubated for 30 minutes at 37°C with aerolysin in the first well of a microtiter plate to investigate the ability of glycophorins to inhibit aerolysin mediated hemolysis.

In a further set of experiments human glycophorin was incubated with either trypsin (1 μg/ml final) or proteinase K (10 μg/ml final). Samples were collected 0, 5, 15, 30, 45 and 60 minutes after the addition of protease, and further digestion was inhibited by the addition of soybean trypsin inhibitor (10 μg/ml final) or PMSF (1 mM final) respectively. The samples were preincubated with aerolysin (10 μg glycophorin to 10 μg aerolysin in each lane) in the first wells of a microtiter plate as before and serially diluted and titered as described above. Samples were also added to 4 x sample buffer, boiled, separated on SDS-PAGE and Coomassie stained.

Phosphatidylinositol-specific Phospholipase C Treatment of Erythrocytes and RBCMs

Erythrocytes were prepared from fresh rat blood or outdated human blood by washing with PBS as described. 100 mU PI-PLC (Boehringer Mannheim) were added to

1 ml of a 10% suspension of these erythrocytes in PBS and incubated at 37°C for 30 minutes. Cells were pelleted and the supernatant was collected. Samples of both cells and supernatant were run on SDS-PAGE for blotting and staining, and the cells were used in aerolysin titres.

Radiolabeling Proaerolysin

Preparation of ^{35}S-Methionine Labeled Proaerolysin

An overnight culture of *A. hydrophila* strain 2H$^-$ carrying pKWB5.3 was subcultured 1:100 into 20 ml of fresh LB / Davis / glucose media containing 50 μg/ml ampicillin and 0.05 μM/ml pirazmonam. After four hours, when the OD_{600} reached 3.5, the culture was divided into two sterile 10 ml tubes and pelleted for 5 minutes in a bench centrifuge. Supernatants were collected and each cell pellet was resuspended in 10 ml of fresh modified Riddles media, containing 0.42% glycerol, 1 mM IPTG and antibiotics as in the growth media above, prewarmed to 28°C. Radiolabel (1 mCi of ^{35}S-Met) was also added to one tube. The cultures were incubated for a further 90 minutes in a rotatory incubator at 28°C before the cells were pelleted for 15 minutes at 3000 rpm in a Beckman JA-20 rotor. Supernatants were collected and phenanthroline and benzamidine were each added to a 1 mM final concentration. The unlabeled supernatant and the initial 20 ml culture supernatant were titred as described previously.

Radio-iodination of Proaerolysin

Proaerolysin was labeled with ^{125}I as described previously (Howard and Buckley, 1982). Iodogen (Sigma) (30 µl of a 1 mg/ml solution in $CHCl_3$) was dried onto the sides and bottom of a small tube under N_2. The reaction was initiated by the addition of 800 µl of proaerolysin (OD_{280} between 0.5 and 1.0 in 20mM HEPES, pH 7.4) followed by 0.5 mCi $Na^{125}I$ (approximately 5 µl). The reaction was allowed to proceed for 15 minutes on ice with occasional agitation before the remaining free iodine was quenched by transferring the mixture to a fresh tube containing 20 µl of a 0.3 mg/ml tyrosine solution. After a further 5 minutes on ice, the tube contents were loaded onto a PD-10 column (Pharmacia) and the radiolabeled protein was subsequently eluted in three 700 µl fractions free of the unbound ^{125}I. The OD_{280} was determined and 5 µl of each fraction were counted to determine the specific activity.

Investigating the Aerolysin Receptor in Other Cell Lines

A number of other cell and tissue types from various sources were prepared, solubilized in sample buffer, run on SDS-PAGE, blotted and probed using the proaerolysin sandwich Western blot technique described. These cells were prepared by washing three or more times with PBS in the same way as erythrocytes. Anchorage-dependent cell lines were first released from the substrata by treating for 5 minutes with trypsin before washing.

Mouse erythrocytes, macrophages (supplied by Siobhan Cowley), T-cell line EL-4, myeloma cell line, B-cell line, and fibroblast cell line L-929 (supplied by Rob

Beecroft) were all tested as well as human erythrocytes, rockfish erythrocytes (supplied by Dr. Mommsen), rat macrophages (Siobhan Cowley), trypanosomes, African Green monkey COS-1 kidney cell line and *Isochyris* cells (a marine invertebrate; all supplied by Rob Beecroft).

RESULTS

Identification of the 47 kDa Aerolysin Receptor Blot Band

It has been previously demonstrated that the aerolysin receptor in rat erythrocytes is a glycoprotein (Howard and Buckley, 1982; Garland and Buckley, 1988). To determine whether the rat erythrocyte membrane contained a single high-affinity receptor species, and to derive an estimate of the molecular mass of the receptor, rat erythrocyte membranes were prepared, solubilized by boiling in sample buffer and separated by SDS-PAGE. The membrane proteins were transferred to a nitrocellulose membrane and this blot was probed with proaerolysin at 1 μg/ml (2×10^{-8} M). Any toxin which had bound to the blot was detected by using a mouse anti-aerolysin monoclonal antibody, followed by an anti-mouse antibody conjugated with either alkaline phosphatase or horseradish peroxidase. Corresponding samples on separate gels which had been run under the same conditions were stained with Coomassie blue, and by the periodate / Schiff's method.

From the blot result in Figure 3 it can be seen that the rat erythrocyte membrane contains a single major species, migrating with an apparent molecular weight of approximately 47 kDa on a SDS-polyacrylamide gel which binds aerolysin under these conditions. On some blots a second, more diffuse band was sometimes seen which migrated with an apparent molecular weight of 30 kDa. This band may be related to the 47 kDa band, representing a partial breakdown product of some kind, or it may be a form of the same polypeptide that has been post-translationally processed in some different way. Alternatively it may represent a different aerolysin-binding component of the rat erythrocyte membrane with slightly lower affinity. The periodate / Schiff's gel staining

pattern (Figure 4) indicated that the 47 kDa aerolysin-binding protein (47 kDa ABP) protein does not correspond to any one of the major glycoproteins of the rat erythrocyte membrane, as these species migrate with apparent molecular weights of approximately 39 kDA and 76 kDA. After periodate / Schiff's staining no glycoprotein band was visible at 47 kDa, even though twice as much rat RBCM material was loaded as was used in the blot lane. In the Coomassie stained gel (Figure 5) many proteins were visible, but, although there were a number of bands visible around 47 kDa none of these was very pronounced. These results suggest that either the receptor is not present in high copy number, or it is not easily stained by either of these methods.

Aerolysin Receptor Proteins in Other Cell Lines

Rat erythrocytes are amongst the most sensitive to aerolysin while human erythrocytes are amongst the least sensitive. It has been reported that mouse erythrocytes have a similar sensitivity to rat (Bernheimer and Avigad, 1975; Howard and Buckley, 1982), and little is known about the aerolysin sensitivity of non-erythroid cell lines. To investigate whether a high affinity aerolysin-binding protein could be detected in cell types other than rat erythrocytes by using the blotting method, a number of other cell and tissue types were prepared. These samples, both from rats and from other species, were washed, solubilized in sample buffer, run on SDS-PAGE, blotted and probed using the proaerolysin sandwich Western blot technique. As can be seen in Figure 3 most of the

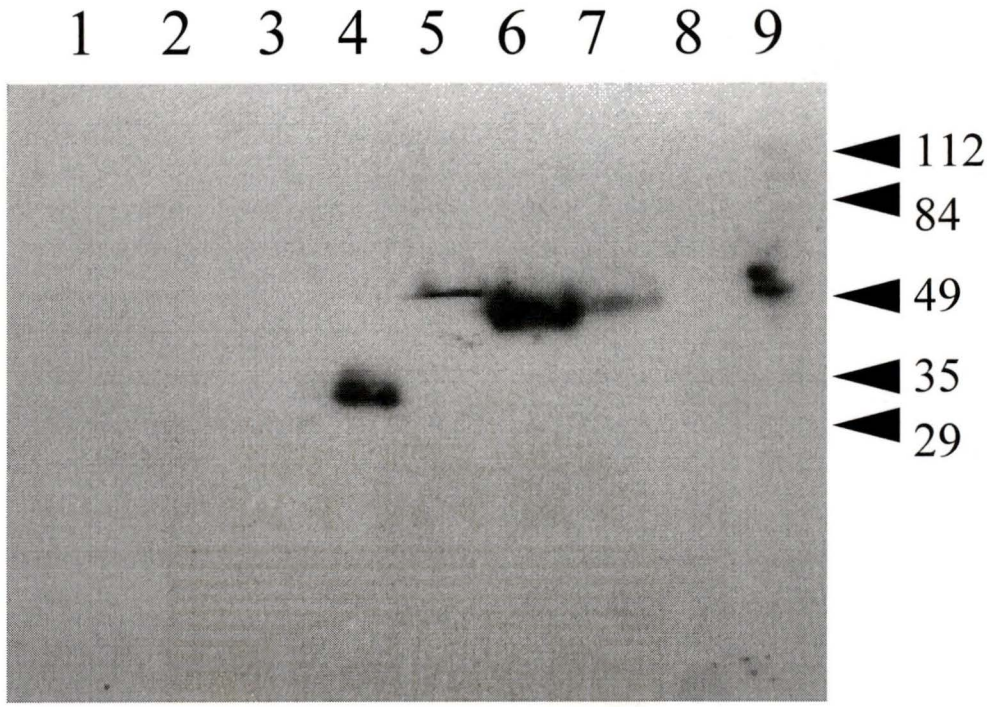

Figure 3: Western blot of a gel showing the aerolysin binding proteins found in a number of eukayotic cell lines. The proteins were transferred to nitrocellulose developed by the ECL protocol, showing aerolysin binding proteins in a number of eukayotic cell lines. Lane 1, Mouse B-cells; lane 2, Mouse macrophage; lane 3, Mouse myeloma; lane 4, Mouse thymoma cells; lane 5, Mouse erythrocyte ghosts; lane 6, Rat erythrocyte ghosts; lane 7, Human erythrocyte ghosts; lane 8, molecular weight standards; lane 9, proaerolysin.

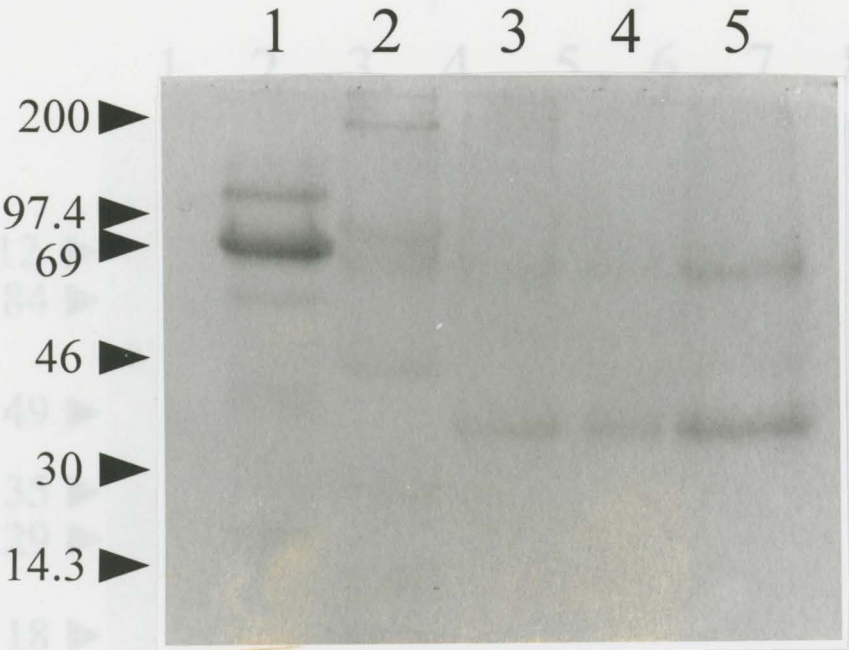

Figure 4: Rat and human erythrocyte glycoproteins visualised by periodate / Schiff's staining of an SDS-PAGE gel. Lane 1, Human glycophorin; lane 2, molecular weight standards (values given in kDa); lane 3, rat erythrocyte ghosts; lanes 4 and 5, rat glycophorin analogues. Lane 5 contains twice the concentration of glycoprotein that is in lane 4.

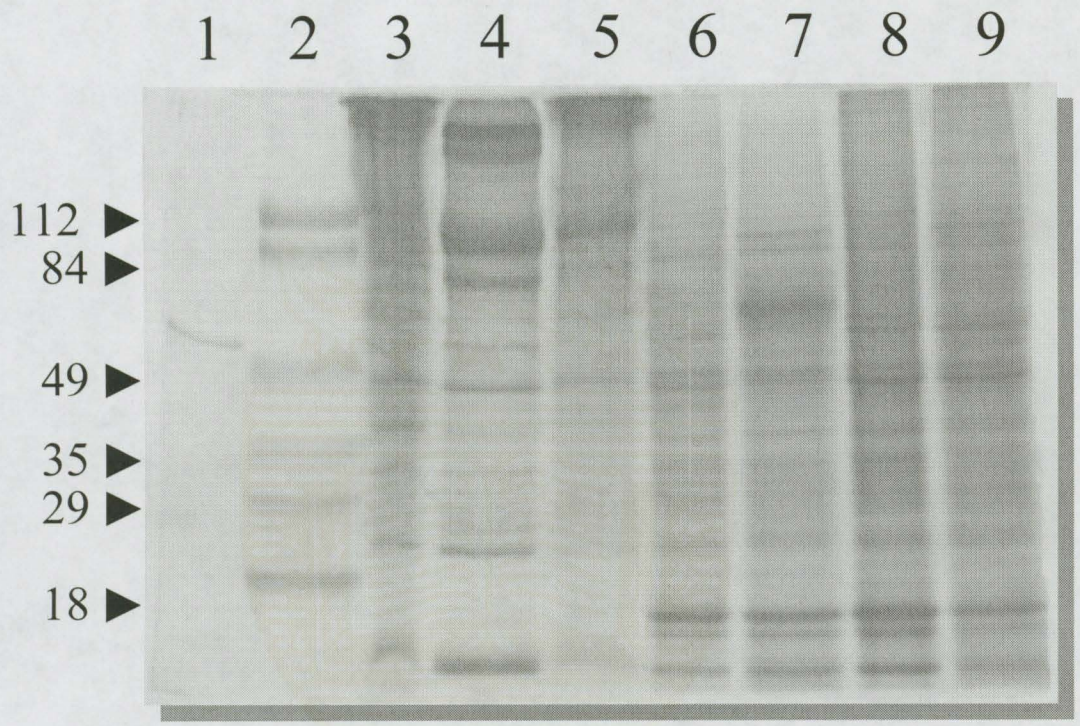

Figure 5: Coomassie stained SDS-PAGE gel showing the proteins found in a number of eukaryotic cell lines. Lane 1, proaerolysin; lane 2, molecular weight standards; lane 3, human erythrocyte ghosts; lane 4, rat erythrocyte ghosts; lane 5, mouse erythrocyte ghosts; lane 6, mouse thymoma cells; lane 7, mouse myeloma; lane 8, mouse macrophage; lane 9, mouse B-cells.

cell lines tested do not possess a single high affinity aerolysin binding species. However the mouse T-cell line EL-4 and mouse erythrocytes both contained a single species which bound aerolysin with high-affinity on the blot. The apparent molecular masses of the aerolysin binding species in the three cell lines (rat erythrocyte, mouse erythrocyte and mouse T-cell) are different. Human erythrocyte membranes do not appear to contain a specific aerolysin binding species. Instead aerolysin appeared to be able to bind to a number of proteins in the human erythrocyte membrane preparation but with a lower affinity than was seen for the rat 47 kDa ABP. One of these human erythrocyte proteins may be to human glycophorin (one of the proteins to which aerolysin binds on a blot of human erythrocyte ghost proteins has a similar mobility to human glycophorin) which has been proposed as a potential aerolysin binding species by Howard and Buckley (1982).

Glycophorin from Human and Rat Erythrocytes

Howard and Buckley (1982) suggested that human glycophorin and its rat analogue may act as aerolysin receptors on erythrocyte membranes. In order to determine whether this glycoprotein was able to bind aerolysin, the method of Marchesi and Andrews (1971) was used to prepare glycophorin from both human and rat erythrocyte membranes (prepared as per Dodge *et al.*, 1962; method 1). Although preincubation of aerolysin with a equal concentration of human glycophorin caused inhibition of aerolysin-mediated human erythrocyte hemolysis in a titre (as was previously reported by Garland and Buckley, 1988), human glycophorin immobilized on a nitrocellulose blot

showed no significant aerolysin binding activity when probed with aerolysin using the sandwich Western blot method (results not shown). Rat glycophorin was also able to partially inhibit aerolysin-mediated hemolysis after preincubation of a fivefold or greater molar excess of the membrane protein with aerolysin (Table 2). However, when samples were separated by SDS-PAGE, blotted and probed by the aerolysin sandwich method, no strong receptor band was visible (Figure 6). The 47 kDa band copurified with rat glycophorin through the early stages of the glycophorin purification procedure but was apparently lost into the phenol phase during extraction, whereas glycophorin remained in the aqueous phase (Figure 6).

Treatment of human glycophorin with either trypsin or proteinase K abolished the aerolysin inhibition (Table 2). From the Coomassie stained gels (Figures 7 and 8) it can be seen that the loss of the inhibitory activity coincided with the disappearance of the 79 kDa major glycophorin A band indicating that only intact human glycophorin was able to inhibit aerolysin.

Using Alternate Methods to Verify that Proaerolysin Binds to the 47 kDa Band

In order to confirm that aerolysin was binding specifically and almost exclusively to the 47 kDa ABP it was decided that alternative methods should be used to investigate the binding pattern of aerolysin to rat erythrocyte membrane proteins on a nitrocellulose blot. Proaerolysin was radiolabeled with ^{125}I or with ^{35}S-methionine. Rat erythrocyte membrane proteins, dissolved in sample buffer, were separated by SDS-PAGE and blotted onto nitrocellulose. The blots were probed with either of the radiolabeled

	μg glycoprotein in pre-incubation	Number of wells cleared after 60 minutes at 37°C
Human Glycophorin (incubations with 10μg aerolysin)		
	0μg	10 wells
	5μg	6 wells
	50 μg	5 wells
Protease treated human glycophorin* (10μg aerolysin)	50 μg	10 wells
Rat glycophorin (incubations with 1 μg aerolysin)		
	0 μg	8 wells
	5 μg	6 wells
	20 μg	4 wells

Table 2: Inhibition of aerolysin after preincubation with human or rat glycophorin. Hemolytic titre results.
* After incubation of human glycophorin for 60 minutes with either trypsin or proteinase K, the results were the same.

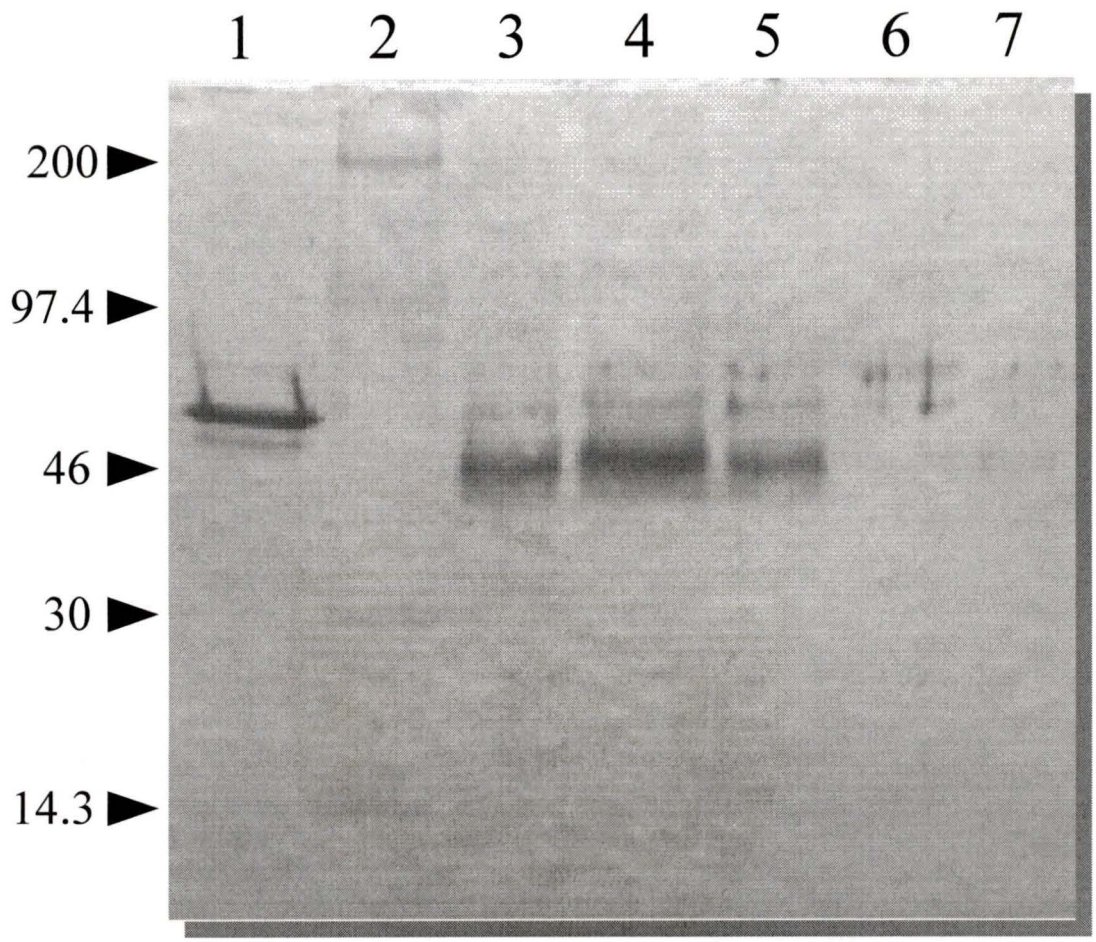

Figure 6: Western blot developed by the alkaline phosphatase method showing the fate of the 47 kDa ABP through the steps for the preparation of the rat erythrocte glycophorin analogue. Lane 1, proaerolysin; lane 2, rainbow molecular weight markers (values in kDa); lane 3, rat erythrocyte ghost proteins; lane 4 and 5, LIS soluble fraction after centrifugation 20 and 10 μl repectively; lane 6, LIS insoluble pellet; lane 7 rat glycophorin.

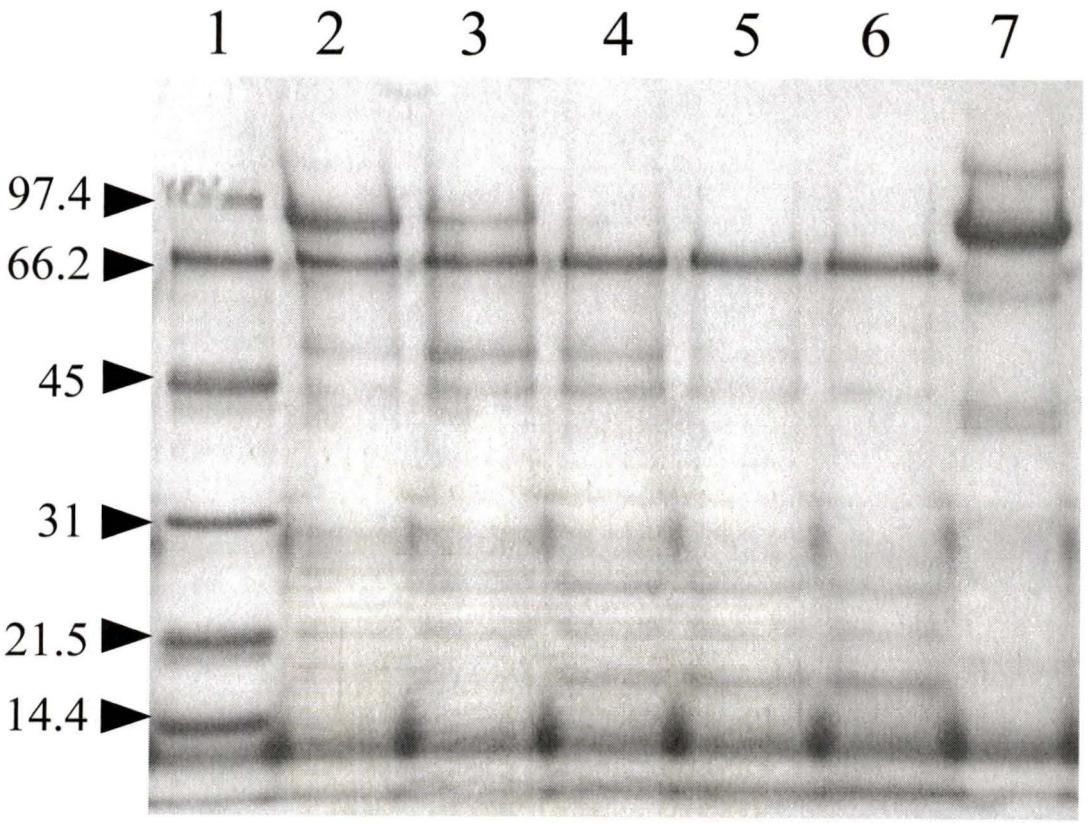

Figure 7: Coomassie stained SDS-PAGE gel showing the trypsin sensitivity of human glycophorin. Lane 1, protein molecular weight standards (given in kDa); lane 2, sample taken immediately after the addition of trypsin; lane 3, t=5 minutes; lane 4, t=15 minutes; lane 5, t=30 minutes; lane 6, t=60 minutes; lane 7, untreated human glycophorin.

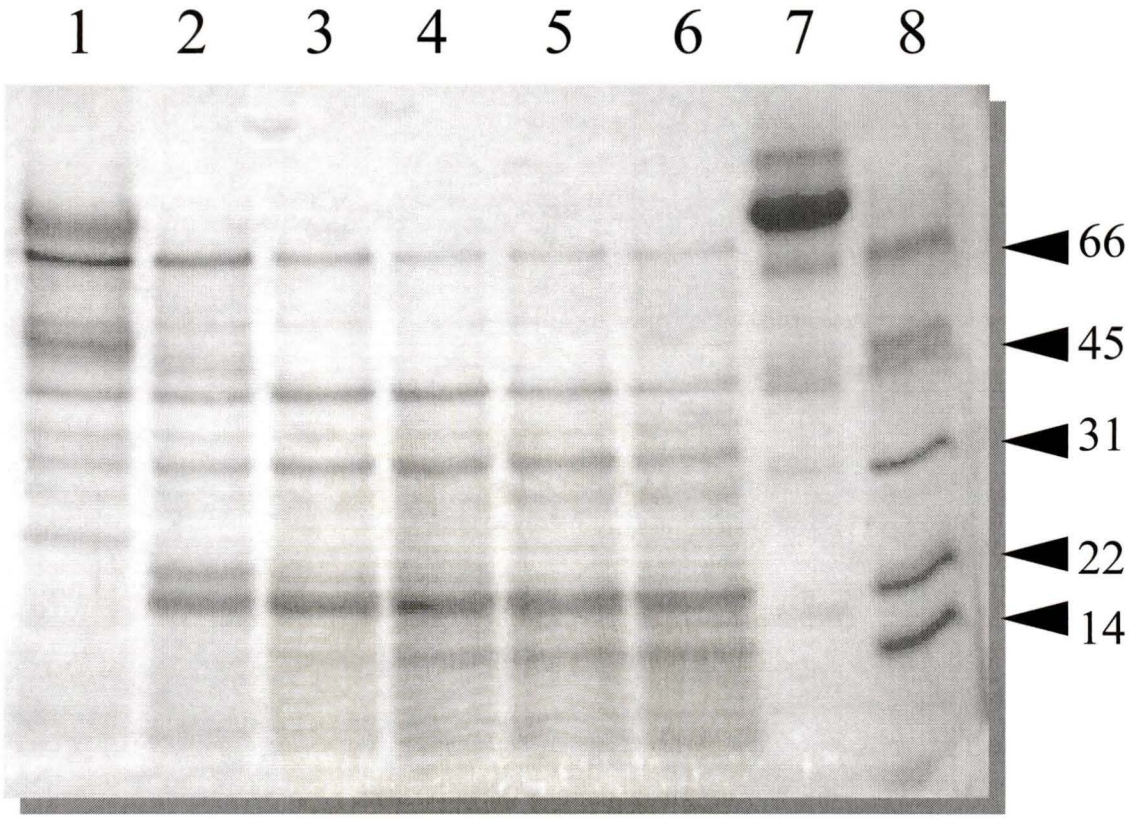

Figure 8: Coomassie stained SDS-PAGE gel showing the effect of proteinase K on human glycophorins. Lane 1, sample taken immediately after the addition of trypsin; lane 2, t=5 minutes; lane 3, t=15 minutes; lane 4, t=30 minutes; lane 5, t=45 minutes; lane 6, t=60 minutes; lane 7, untreated human glycophorin; lane 8, protein molecular weight standards (in kDa).

proaerolysins at 1 μg/ml in PBS-T. For the ^{35}S-methionine labeled proaerolysin, 5mM cold methionine was also added to the PBS-T to compete out non-specific binding by free ^{35}S-methionine contaminating the preparation. After washing with PBS-T until the amount of radioactivity in the washing solution fell to a constant low level, the blots were dried and exposed to film. The ^{35}S-methionine labeled proaerolysin bound specifically to a band which migrates at 47 kDa on the blot (Figure 9) in agreement with the results obtained by the aerolysin sandwich Western blot method.

The ^{35}S-labeled 47 kDa bands from these blots were cut out and counted in order to determine how much proaerolysin was bound to different concentrations of rat erythrocyte ghost material and to allow a rough calculation of copy number for the 47 kDa ABP. A value of 10^4 copies per erythrocyte cell was derived, an order of magnitude lower than the copy number estimated by Howard and Buckley earlier (1982) using a ^{125}I labeled proaerolysin preparation binding to intact erythrocytes. This discrepancy may result from a change in the receptor binding specificity of aerolysin resulting from ^{125}I labeling of the protein (Fraker and Speck, 1978; Jakki Cooney, personal communication).

For ^{125}I-labeled proaerolysin, two contrasting results were obtained. In the first blot probed, the ^{125}I-labeled proaerolysin bound to a higher molecular weight species (76 kDa; Figure 9) which may correspond to the rat erythrocyte glycophorin analogue. When the experiment was repeated with freshly ^{125}I labeled proaerolysin the radioactivity was found to be bound to the 47 kDa band (not shown) and not to the higher weight band seen in the previous experiment. Since the iodination method which we used is specific for tyrosine residues this result may suggest that there is a tyrosine residue at or near the

receptor binding site in the aerolysin molecule. Labeling of the protein, which introduces a relatively large, electronegative iodide species, may have affected the binding specificity of proaerolysin. It may be that subtle differences in the conditions for the labeling reaction resulted in different tyrosine residues in the aerolysin molecule being preferentially labeled by the ^{125}I. Alternatively the labeling reaction may have proceeded such that the extent of labeling of different tyrosines varied between the two preparations. For example, in the first experiment, a tyrosine at or near the binding site might have been more heavily labeled, altering the binding specificity of the proaerolysin so that it can now bind to the rat glycophorin analogue. In the second experiment labeling of this tyrosine may not have occurred to any significant extent.

Other methods to stain or identify the aerolysin binding band in gels and in blots were also tried. Blots were probed with biotinylated lectins (wheat germ agglutinin and concanavalin A), as described in methods, but this technique did not detect the 47 kDa band. Similarly attempts to biotinylate or fluorescence label glycoproteins in the membrane preparation and visualize the receptor protein on a blot were also unsuccessful. None of these techniques seemed to have the necessary degree of sensitivity since in each case only the major erythrocyte membrane glycoproteins (i.e. those visualized by periodate / Schiff's staining) were ever visualized.

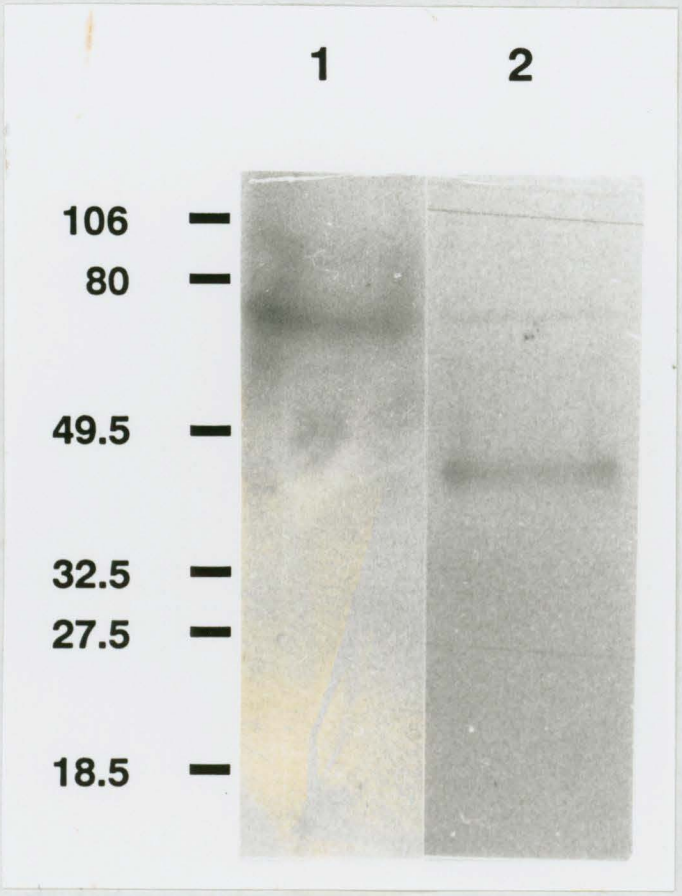

Figure 9: Detecting the rat erythrocyte 47 kDa ABP using radiolabelled aerolysin. Erythrocyte membrane proteins were separated by SDS-PAGE, blotted onto nitrocellulose and probed with radiolabelled aerolysin as described in methods. Lane 1, radioiodinated proaerolysin; lane 2, ^{35}S-labelled proaerolysin. The positions of molecular weight markers are indicated.

Detergent Extraction of Proteins from Erythrocyte Membranes

In order to develop a purification protocol for the 47 kDa ABP from rat erythrocyte membranes it was first necessary to find an effective method to solubilize it. A number of detergents were tested, including Triton-X-100, Tween-20, octyl glucoside and octyl Pol (which were tried in both salt free and 0.2 M NaCl solution) as well as CHAPS and SLS. Samples of the extract supernatants and residual pellets were separated by SDS-PAGE and blotted onto nitrocellulose. The blots were developed by the aerolysin sandwich Western method and the relative amounts of receptor in the supernatants and the pellets were estimated by comparing the intensity of the 47 kDa bands in their respective lanes (Figure 10). It was determined that CHAPS, SLS and Triton-X-100 were all effective at solubilizing the 47 kDa band from rat erythrocytes (more than half of the original aerolysin binding activity from the ghosts was found in the supernatant). Tween-20 and octyl Pol were less effective, and octyl glucoside in water did not solubilize any of the 47 kDa band, although it did solubilize a number of other proteins from the erythrocyte membranes (Figures 10 and 11). Based on these results either CHAPS or Triton-X-100 was used to extract the 47 kDa protein in subsequent experiments.

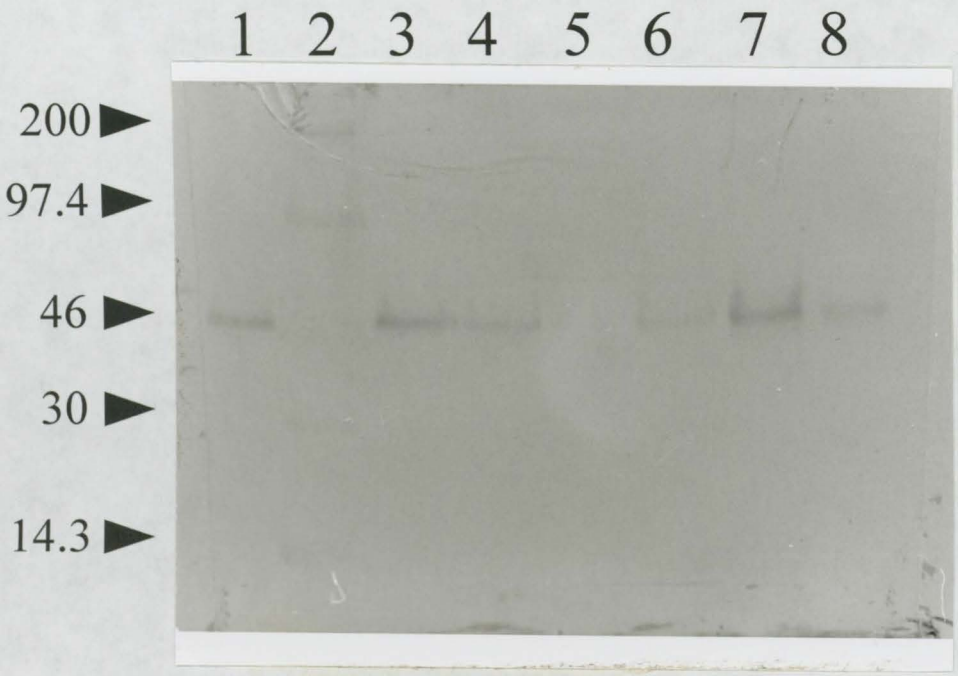

Figure 10: Detergent extraction of rat erythrocyte ghost proteins. Western blot of a gel identical to the one in Figure 11. Lane 1, rat erythrocyte ghost proteins; lane 2, protein molecular weight standards (given in kDa); lane 3, Triton in dH_2O extract; lane 4, Triton in 0.8M NaCl extract; lane 5, octyl glucoside in dH_2O extract; lane 6, octyl glucoside in 0.8M NaCl extract; lane 7, CHAPS in 0.8M NaCl extract; lane 8, LIS in 0.8M NaCl extract.

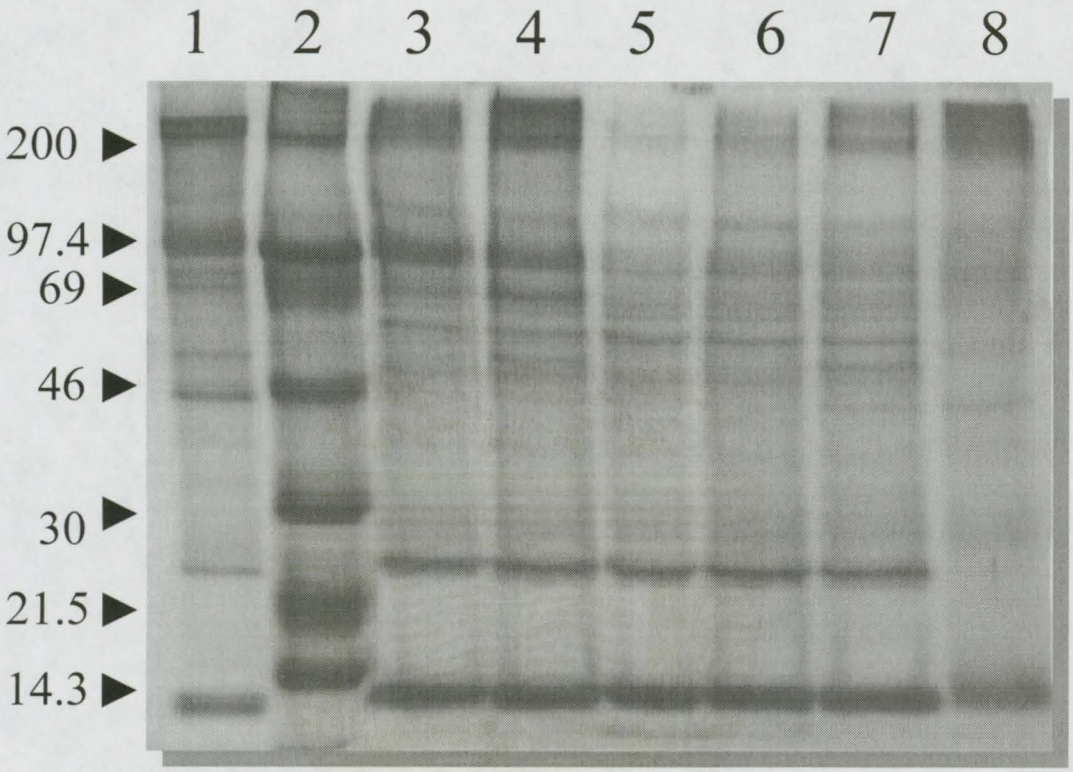

Figure 11: Detergent extraction of rat erythrocyte ghost proteins. Coomassie stained SDS-PAGE gel. Lane 1, rat erythrocyte ghost proteins; lane 2, protein molecular weight standards (given in kDa); lane 3, Triton in dH_2O extract; lane 4, Triton in 0.8M NaCl extract; lane 5, octyl glucoside in dH_2O extract; lane 6, octyl glucoside in 0.8M NaCl extract; lane 7, CHAPS in 0.8M NaCl extract; lane 8, LIS in 0.8M NaCl extract.

Column Purification of the 47 kDa Aerolysin Binding Protein

In order to further purify the solubilized 47 kDa band, a chromatography column was used. The binding of the 47 kDa band, solubilized in CHAPS or Triton-X-100, to a number of different chromatography matrices was investigated. DEAE-sepharose, hydroxylapatite, wheat germ agglutinin (WGA) sepharose and immobilized proaerolysin (bound to either CNBr activated sepharose or activated thiol-sepharose) were tested. The aerolysin binding protein bound to the WGA-sepharose, and could then be eluted by passing 0.1 M N-acetyl glucosamine through the column, suggesting that it has surface-exposed N-acetyl glucosamine or sialic acid residues. The 47 kDa ABP also bound to DEAE-sepharose at pH 8.0, but not at pH 7.4. It could be subsequently eluted from the DEAE-sepharose matrix by low concentrations of NaCl (<0.1 M). Aerolysin was immobilized onto chromatography resin to facilitate the construction of an affinity column for purification of the receptor. When a receptor-containing solution was passed over this matrix, the receptor was apparently retained on the column. Both high and low pH (pH 10 and 4), and high salt (1 M NaCl) were tested as a means of releasing the bound receptor. None of these methods were successful, as determined by running eluate samples on SDS-PAGE, blotting and probing by the aerolysin sandwich method. Either the binding of the receptor to aerolysin is so strong that the interaction is not broken under these conditions, or the liberated receptor could no longer adopt an aerolysin-binding conformation. Consequently, this method was not further pursued. The 47 kDa protein did not bind to hydroxylapatite under the conditions tested.

Purification Protocol for the 47 kDa Aerolysin Receptor

Based on the detergent solubility and the matrix binding properties described, a purification protocol was derived which involved extraction in Triton-X-100 and DEAE-chromatography followed by WGA-chromatography as described in methods and summarized in Figure 12. Figures 13 and 14 respectively show silver stained and Western blotted fractions collected at each stage through this purification protocol.

By using the acetone precipitation method, it was possible to concentrate the WGA eluate receptor-containing fractions 8 to 10-fold. This made it possible to load sufficient purified material onto a gel to allow the receptor band to be visualized by Coomassie staining the PVDF blot. This simplified the preparation of a sample for N-terminal sequencing.

N-terminal Sequence of Aerolysin Receptor Protein from Rat Erythrocyte Membranes

The N-terminal sequencing and total amino acid composition of samples of the purified receptor protein on PVDF were performed by Sandy Kielland. The procedure gave us an N-terminal sequence of 25 amino acids which was used to search protein sequence data bases through Blast for homologous proteins (Figure 15). The best match (at 66% homology) was with the N-terminus of an ADP-ribosyltransferase from the membrane of rabbit muscle cells (Zolkiewska *et al.*, 1992). Significant homology

Purification of the Rat Erythrocyte Aerolysin Receptor

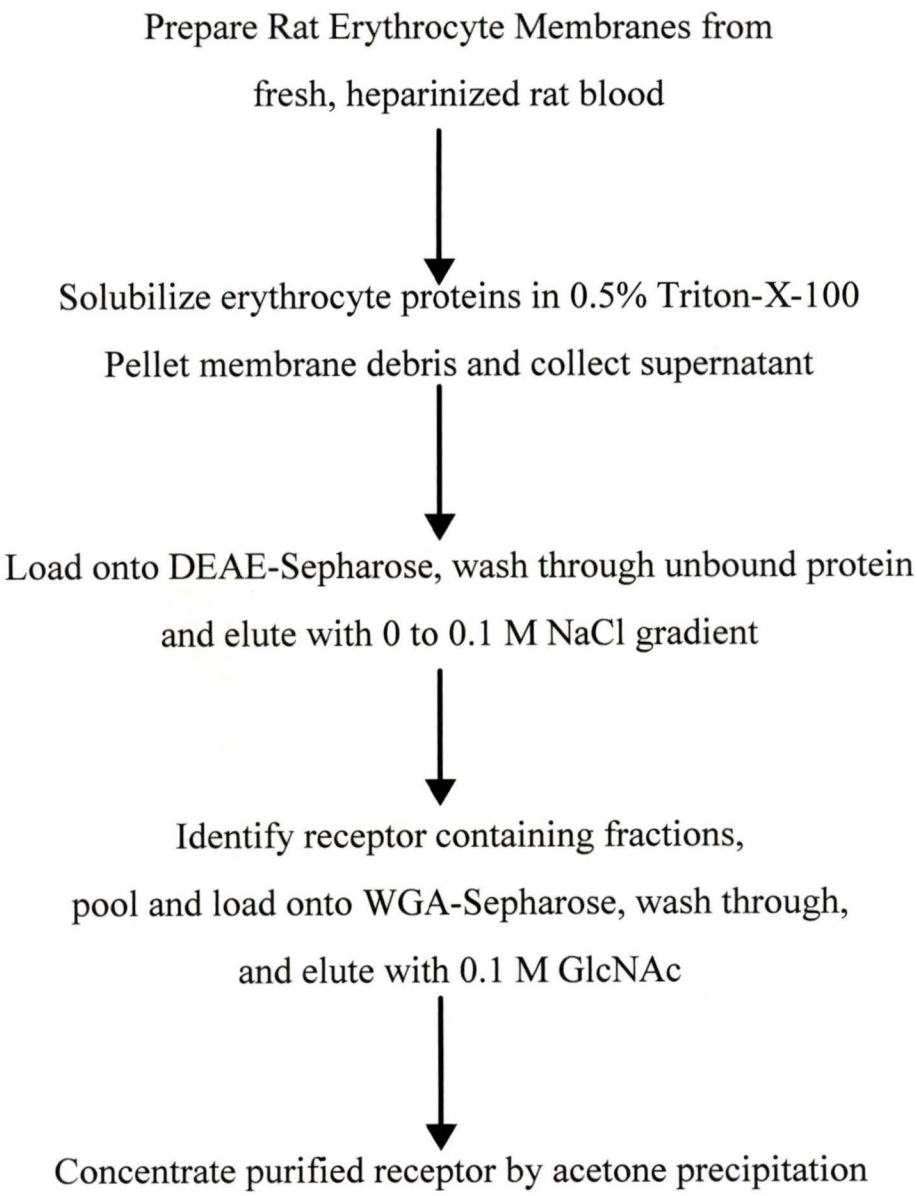

Figure 12: Summary of the purification scheme developed for the rat erythrocyte aerolysin receptor.

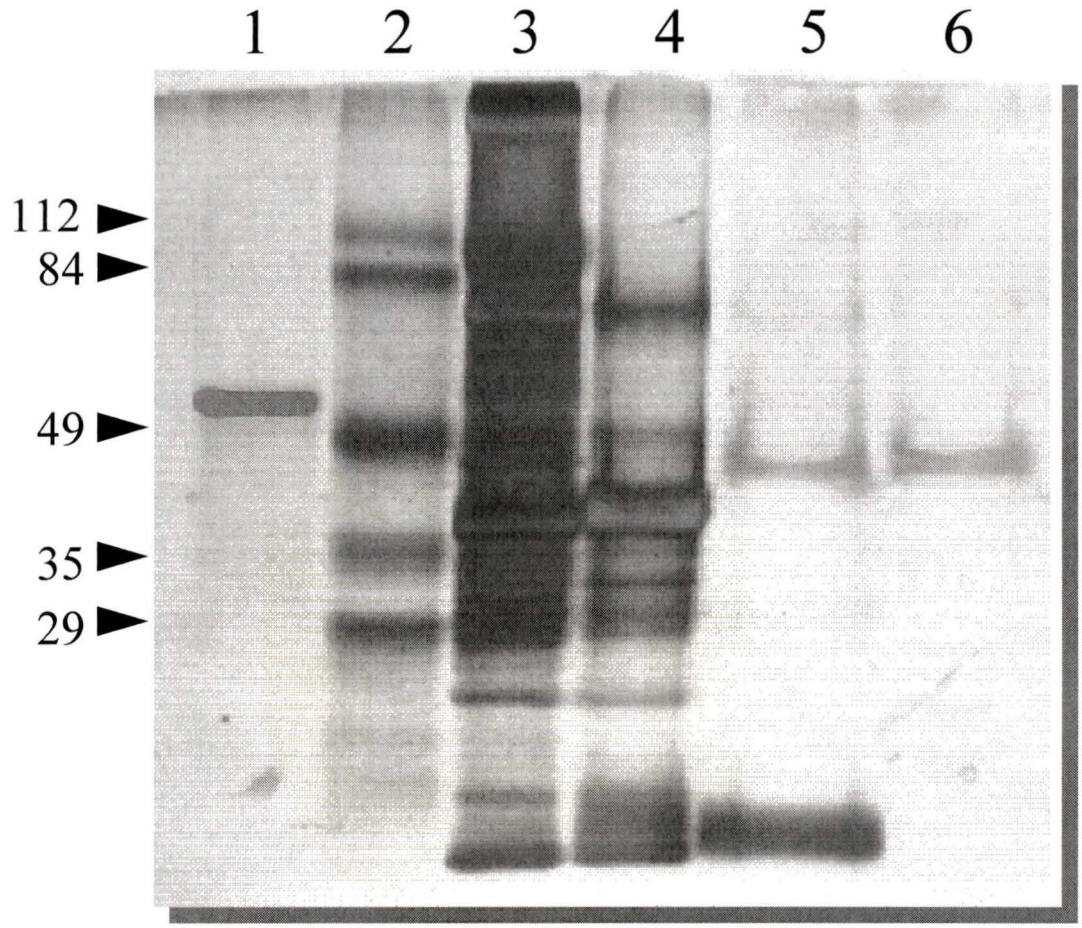

Figure 13: Silver stained SDS-PAGE gel showing the steps in the purification of the 47 kDa ABP from the rat erythrocyte membrane. Lane 1, proaerolysin; lane 2, molecular weight standards (weights given in kDa); lane 3, rat erythrocyte ghost proteins; lane 4, Triton extract of rat erythrocyte ghost proteins; lane 5, pooled ABP containing DEAE-sepharose column eluate fractions; lane 6, ABP containing eluate fraction from the WGA-sepharose column.

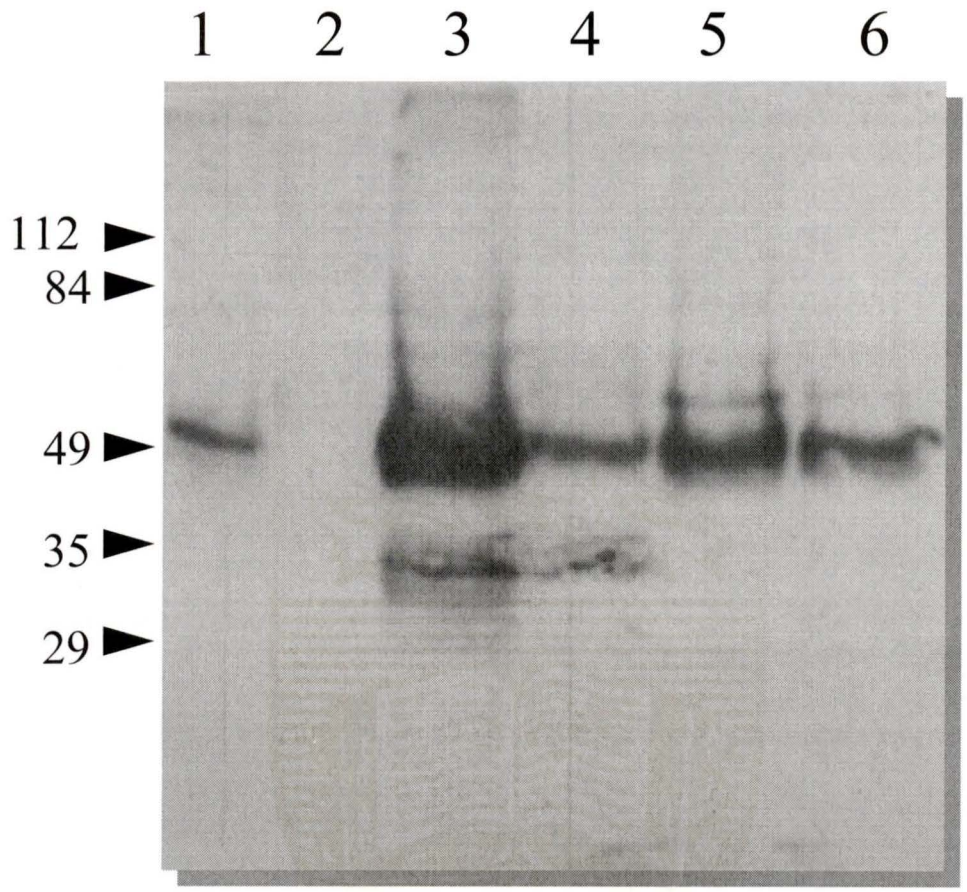

Figure 14: Western blot developed by the ECL method showing the steps in the purification of the 47 kDa ABP from the rat erythrocyte membrane. Lane 1, proaerolysin; lane 2, molecular weight standards; lane 3, rat erythrocyte ghost proteins; lane 4, Triton extract of rat erythrocyte ghost proteins; lane 5, pooled ABP-containing DEAE-sepharose column eluate fractions; lane 6, ABP-containing eluate fraction from the WGA-sepharose column.

(>50%) was also seen with the RT6 family of T-cell marker proteins from the rat and mouse (Koch *et al.*, 1990; Haag *et al.*, 1994)

PI-PLC Treatment of Erythrocytes and RBCM from Rat and Human

It has been reported that the RT6.1, RT6.2 of rat T-cells and a number of eukayotic ADP ribosyltransferase proteins are all attached to plasma membranes via glycosylphosphatidyl inositol (GPI) anchors, some of which can be easily and specifically cleaved enzymatically using phosphatidylinositol specific phospholipase C (PI-PLC; Ikezawa *et al.*, 1976). We used this enzyme to treat both rat and human erythrocytes and rat RBCM. Samples of the treated rat erythrocytes, and the resulting supernatants, were run on SDS-PAGE and blotted or Coomassie stained (Figures 16 and 17). As shown in Figure 17, the 47 kDa band almost disappeared from the rat RBCM lane but was clearly present in the supernatant. In the control sample (no enzyme added), all of the binding activity remained in the erythrocyte fraction. When the treated and control rat erythrocytes were used in aerolysin titres the treated cells showed significantly decreased sensitivity to aerolysin hemolysis (Table 3). At early titre times (5 to 10 minutes) the difference between treated and untreated lanes was greatest at 3 to 4 wells (suggesting sensitivity was reduced 10 fold). After a full 1 hour the difference was reduced to 2 to 3 wells, indicating a 4 to 8-fold decrease in sensitivity. Enzymatic treatment of human erythrocytes did not result in a change in their aerolysin sensitivity (Table 3) indicating that aerolysin does not bind to these cells via a GPI anchored protein.

Supernatants derived from PI-PLC treatment of rat erythrocytes and rat RBCM were used in aerolysin titres to determine whether the solubilized receptor was able to

Type of Erythrocytes Titred	Number of wells cleared at 37°C	
	After 5 minutes	After 60 minutes
Rat Control	7	14.5
Rat PI-PLC treated	3.5	12.5
Human Control	4	10
Human PI-PLC Treated	4	10

Table 3: Effect of phosphatidylinositol specific phospholipase C on the hemolytic sensititvity of rat and human erythrocytes to aerolysin. 5 μg aerolysin was used in each lane.

Rat erythrocyte aerolysin receptor	FSAEVDVDLTPGSFDDQYRG-LKIVLYE
Rabbit skeletal muscle ADP-RT [a]	FSQETPLDMAPASFDDQYVGCAAAMTAA
Human skeletal muscle ADP-RT [b]	FSQEIQLDMALASFDDQYAGCAAAMTAA
Rat T-cell RT6.1 [c]	LTGPLKLDTAPNAFDDQYEGCVNKMEEK
Rat T-cell RT6.2 [d]	LTGPLMLDTAPNAFDDQYEGCVNKMEEK
Human RT6 [c]	LGQPAKLDMADNVFDDQCDVCVEEMEKR
Mouse T-cell RT6-I [c]	LAVPFMLDMAPNAFDDQYEGCVEDMEKK
Consensus sequence	FS-E--LDMAP-SFDDQY-GC---M--- L- P NA V E

Figure 15: N-terminal sequence homology between the rat erythrocyte aerolysin receptor, skeletal muscle mono-ADP-ribosyl transferases (ADP-RTs) and T-cell surface antigens. a, Zolkiewska *et al.*, 1992; b, Okazaki *et al.*, 1994; c, Haag *et al.*, 1994; d, Takada *et al.*, 1994.

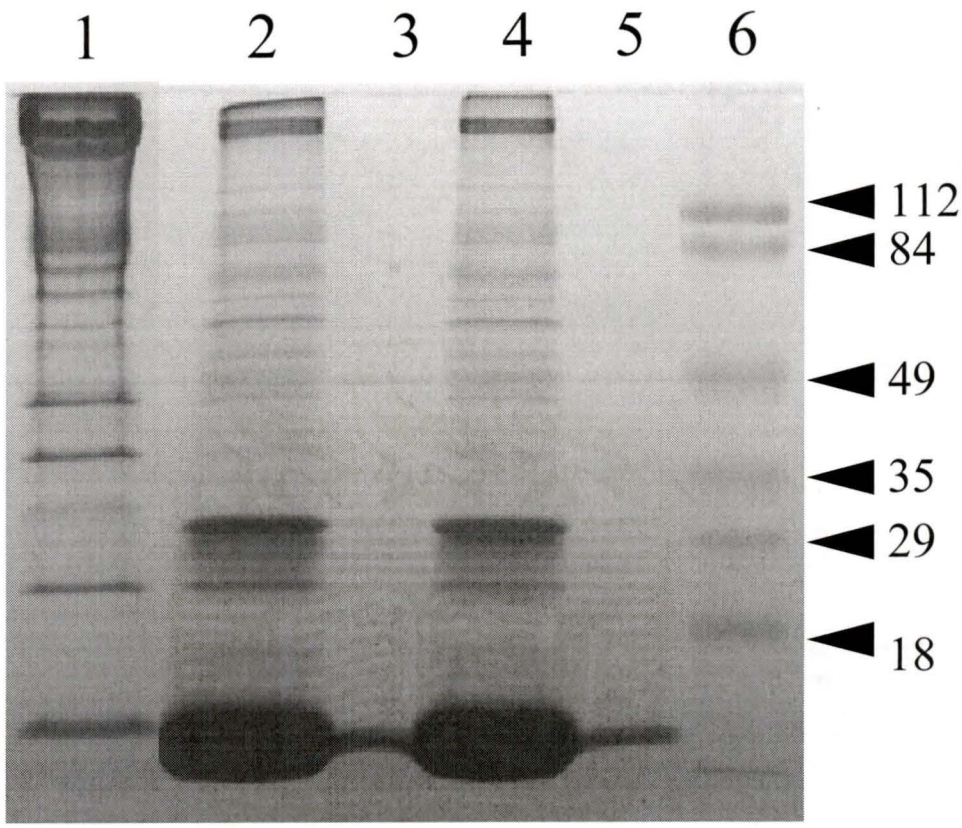

Figure 16: Coomassie stained SDS-PAGE gel showing samples from phosphatidylinositol specific phospholipase C treatment of rat erythrocyte proteins. Lane 1, untreated rat erythrocytes; lane 2, control incubation (no enzyme), rat erythrocyte pellet; lane 3, control incubation, supernatant; lane 4, PI-PLC treated cells, rat erythrocyte pellet; lane 5, PI-PLC treated cells, supernatant; lane 6, protein molecular weight standards (weights given in kDa).

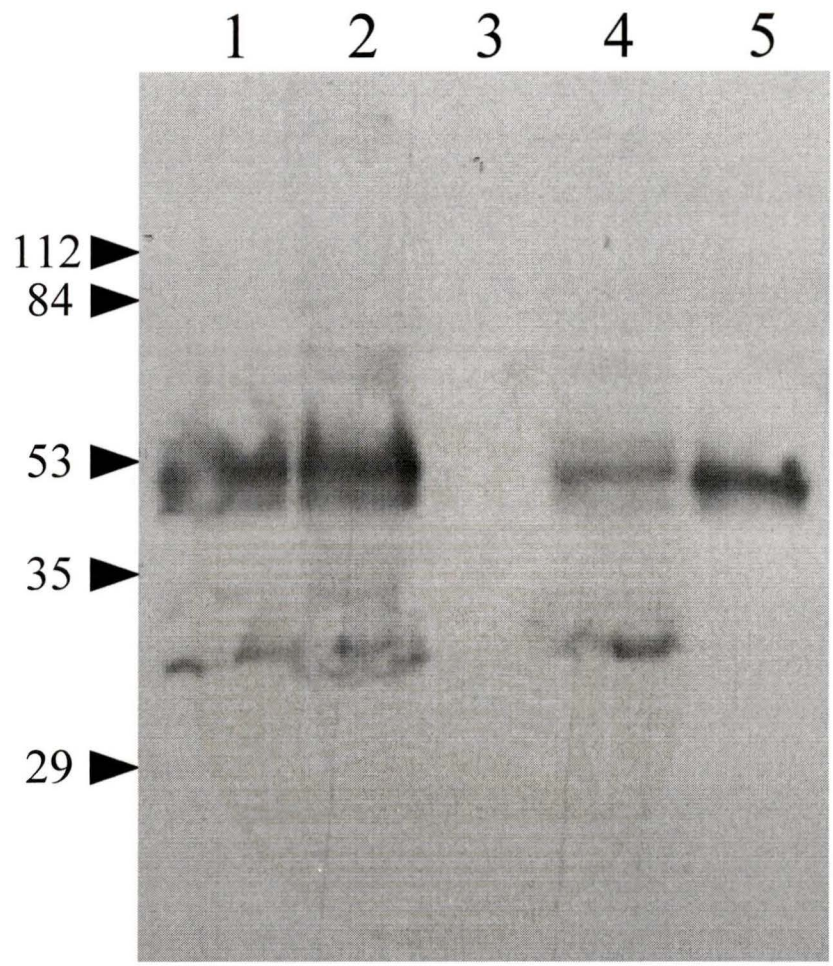

Figure 17: Western blot developed by the ECL method showing samples from phosphatidylinositol specific phospholipase C treatment of rat erythrocyte proteins. Lane 1, untreated rat erythrocytes; lane 2, control incubation (no enzyme), rat erythrocyte pellet; lane 3, control incubation, supernatant; lane 4, PI-PLC treated cells, rat erythrocyte pellet; lane 5, PI-PLC treated cells, supernatant. Molecular weight of protein standards (not shown) are given in kDa.

inhibit aerolysin-mediated hemolysis of untreated human or rat erythrocytes. The results in Table 4 clearly show that the supernatant from the enzyme-treated rat red blood cells or RBCMs was able to inhibit aerolysin hemolysis of either human or rat erythrocytes.

Investigation of the Erythrocyte Receptor for *Staphylococcus aureus* α-toxin

S. aureus α-toxin shares a number of mechanistic features with aerolysin. Both cause target cell lysis by the formation of heptameric transmembrane pores of 1-2 nm diameter (Gouaux *et al.*, 1994) after binding to the eukaryotic cells of sensitive species via interaction with a high affinity receptor, or by non-specific interaction with the bilayer in the case of relatively insensitive human erythrocytes.

To investigate the possibility that the *S. aureus* α-toxin receptor might also be GPI-anchored, the effect of PI-PLC upon rabbit erythrocyte sensitivity to α-toxin was determined. Rabbit erythrocytes are the most sensitive cells known for α-toxin (Cassidy and Harshman, 1976). They were prepared from fresh heparinized rabbit blood as described for rat erythrocytes and treated in the same way with PI-PLC before being titred with 2 hemolytic units of α-toxin (Calbiochem). Table 5 shows that the treatment had no effect upon the sensitivity of the cells to α-toxin, suggesting that the α-toxin receptor is not GPI-anchored in the same way as the rat erythrocyte ABP.

Erythrocyte type used	Supernatant type used	Number of wells cleared after incubation at 37°C for: After 5 minutes	After 60 minutes
Human (using 10 μg aerolysin)	Rat RBC, PI-PLC	3 wells	8.5 wells
	Rat RBC control	4 wells	11 wells
Rat (using 1 μg aerolysin)	Rat RBC, PI-PLC	3 wells	7 wells
	Rat RBC control	6 wells	10 wells
Human (using 10 μg aerolysin)	Rat ghost, PI-PLC	3 wells	8.5 wells
	Rat ghost control	4 wells	11 wells
Rat (using 1μg aerolysin)	Rat ghost, PI-PLC	5 wells	8 wells
	Rat ghost control	6 wells	10 wells

Table 4: Inhibition of aerolysin-mediated lysis of human and rat erythrocytes by supernatants derived from the PI-PLC treatment of intact rat erythrocytes or rat erythrocyte ghosts.

	Number of wells cleared after incubation at 37°C for:		
	5 minutes	60 minutes	300 minutes
PI-PLC treated rabbit erythrocytes	0 wells	4 wells	5 wells
Control rabbit erythrocytes	0 wells	4 wells	5 wells

Table 5: Investigation of the effect of phosphatidylinositol specific phospholipase C treatment on the sensitivity of rabbit erythrocytes to *Staphylococcus aureus* α-toxin.

Protease Sensitivity of the 47 kDa Aerolysin Receptor

The protease sensitivity of the 47 kDa aerolysin receptor was investigated using, trypsin, chymotrypsin (Figures 18 and 19) and proteinase K (Figures 20 and 21). Trypsin treatment of intact erythrocytes caused degradation of the 47 kDa band and reduced the sensitivity of the rat erythrocytes to aerolysin in a hemolytic titre assay (Table 6). Chymotrypsin had no effect upon either the integrity of the blot band or upon the sensitivity of the rat erythrocytes to aerolysin in a titre experiment (Figure 19, Table 6). In contrast, human erythrocytes treated with either trypsin or chymotrypsin exhibited increased aerolysin sensitivity (Table 6). Proteinase K completely degraded the denatured rat erythrocyte receptor protein as can be seen from the blot result in Figure 21. These results demonstrate that the aerolysin receptor is a protein.

De-N-glycosylation of the 47 kDa Aerolysin Receptor

The ability of the aerolysin receptor to bind to WGA-sepharose and be displaced by GluNAc suggests that the protein is glycosylated. Peptide-N-glycosylase F (Oxford Glycosystems) from *Flavobacterium meningosepticum* cleaves N-linked oligosaccharide chains at the terminal N-acetyl-glucosamine β1 aspartyl linkage (Tarentino *et al.*, 1985). To investigate whether the aerolysin receptor was N-glycosylated and whether such a glycan played a role in the interaction with aerolysin binding, rat erythrocyte membranes were treated with this enzyme. This treatment resulted in a shift of the receptor band on a blot from its 47 kDa apparent molecular weight, to give two new bands at approximately

	Protease treatment used	Number of wells cleared after incubation at 37°C for:	
		10 minutes	60 minutes
Rat erythrocytes	Control	4 wells	9 wells
	Trypsin	2 wells	6.5 wells
	Chymotrypsin	4 wells	9 wells
Human erythrocytes	Control	2 wells	9 wells
	Trypsin	5 wells	11 wells
	Chymotrypsin	3 wells	11 wells

Table 6: The effect of serine proteases on the hemolytic sensitivity of rat and human erythrocytes to aerolysin.

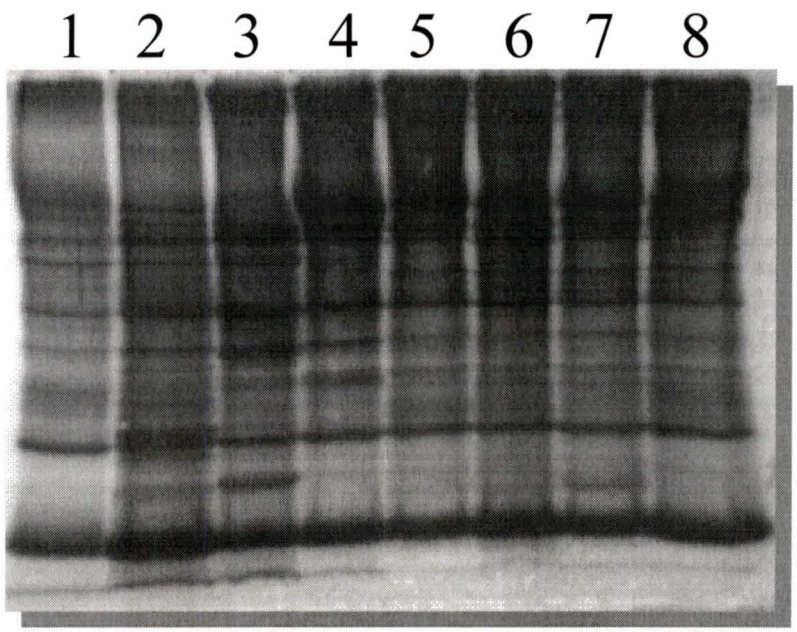

Figure 18: Coomassie stained SDS-PAGE gel showing the effect of serine proteases on rat erythrocyte membrane proteins. Lane 1, untreated human erythrocyte ghost proteins; lane 2, human erythrocytes after trypsin treatment; lane 3, human erythrocytes after chymotrypsin treatment; lane 4, human erythrocyte control (trypsin + trypsin inhibitor); lane 5, rat erythrocyte control; lane 6, rat erythrocytes after chymotrypsin treatment; lane 7, rat erythrocytes after trypsin treatment; lane 8, untreated rat erythrocytes.

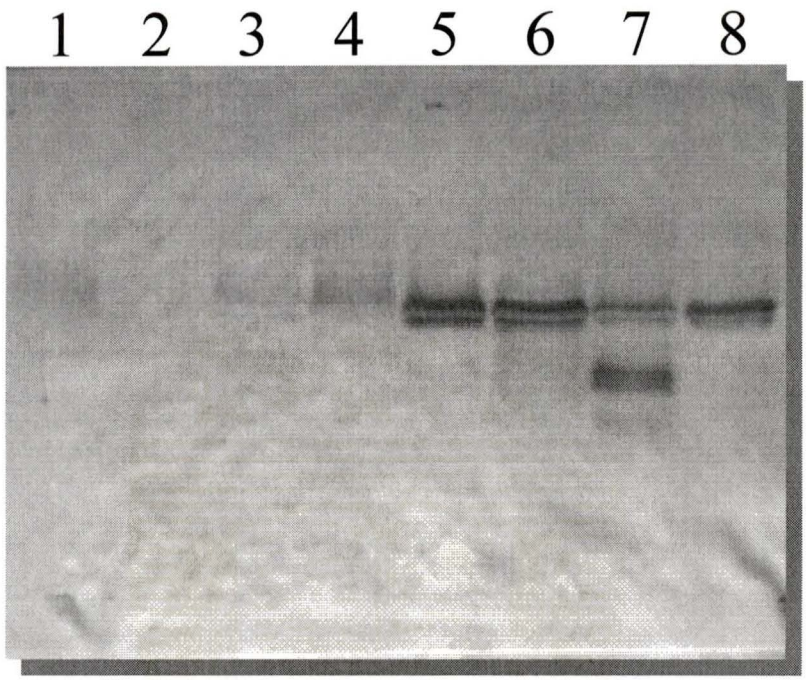

Figure 19: Western blot developed by the alkaline phosphatase method showing the effect of serine proteases on rat erythrocyte membrane proteins. Lane 1, untreated human erythrocyte ghost proteins; lane 2, human erythrocytes after trypsin treatment; lane 3, human erythrocytes after chymotrypsin treatment; lane 4, human erythrocyte control (trypsin + trypsin inhibitor); lane 5, rat erythrocyte control; lane 6, rat erythrocytes after chymotrypsin treatment; lane 7, rat erythrocytes after trypsin treatment; lane 8, untreated rat erythrocytes.

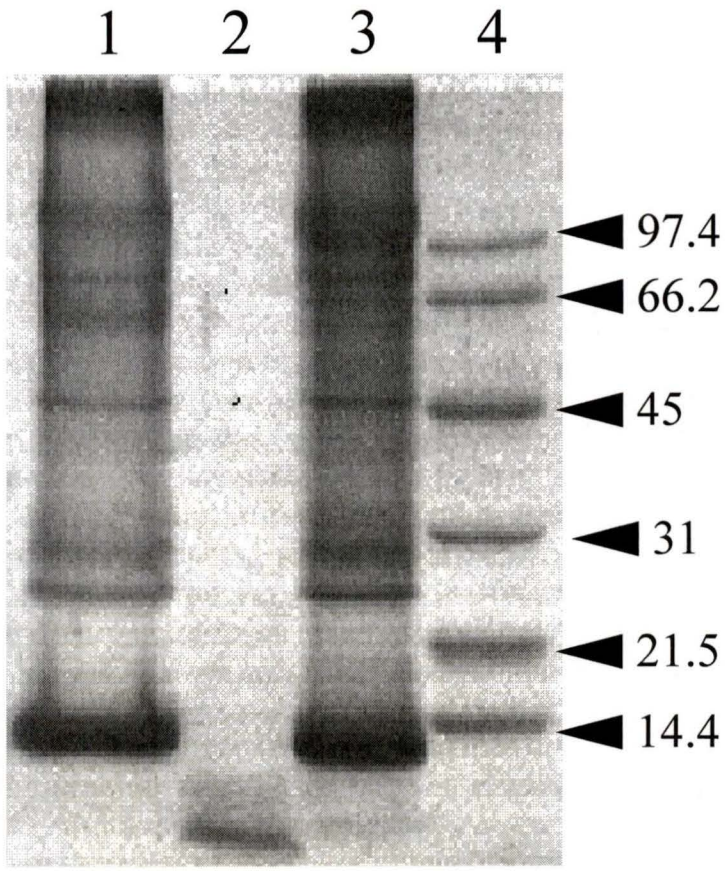

Figure 20: Coomassie stained SDS-PAGE gel showing the effect of proteinase K on rat erythrocyte membrane proteins. Lane 1, untreated rat erythrocyte ghosts; lane 2, proteinase K treated rat erythrocyte ghosts; lane 3, control rat erythrocytes; lane 4, protein molecular weight standards (given in kDa).

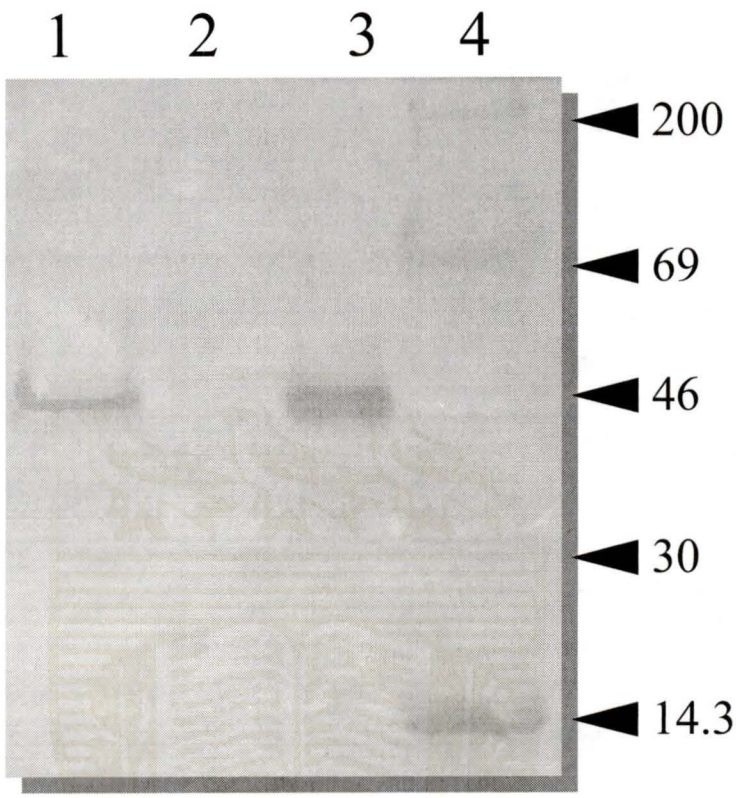

Figure 21: Western blot developed by the alkaline phosphatase method showing the effect of proteinase K on rat erythrocyte membrane proteins. Lane 1, untreated rat erythrocyte ghosts; lane 2, proteinase K treated rat erythrocyte ghosts; lane 3, control rat erythrocytes; lane 4 , protein molecular weight standards (given in kDa).

32 kDa and 38 kDa (Figure 22). Interestingly aerolysin still bound to these new, smaller species, indicating that the N-linked saccharides are not important for aerolysin binding.

This result, taken with the binding of the aerolysin receptor to WGA-sepharose, and subsequent elution with N-acetyl-glucosamine, indicates that the aerolysin receptor contains an N-linked oligosaccharide moiety with N-acetyl-glucosamine and / or sialic acid in its sequence.

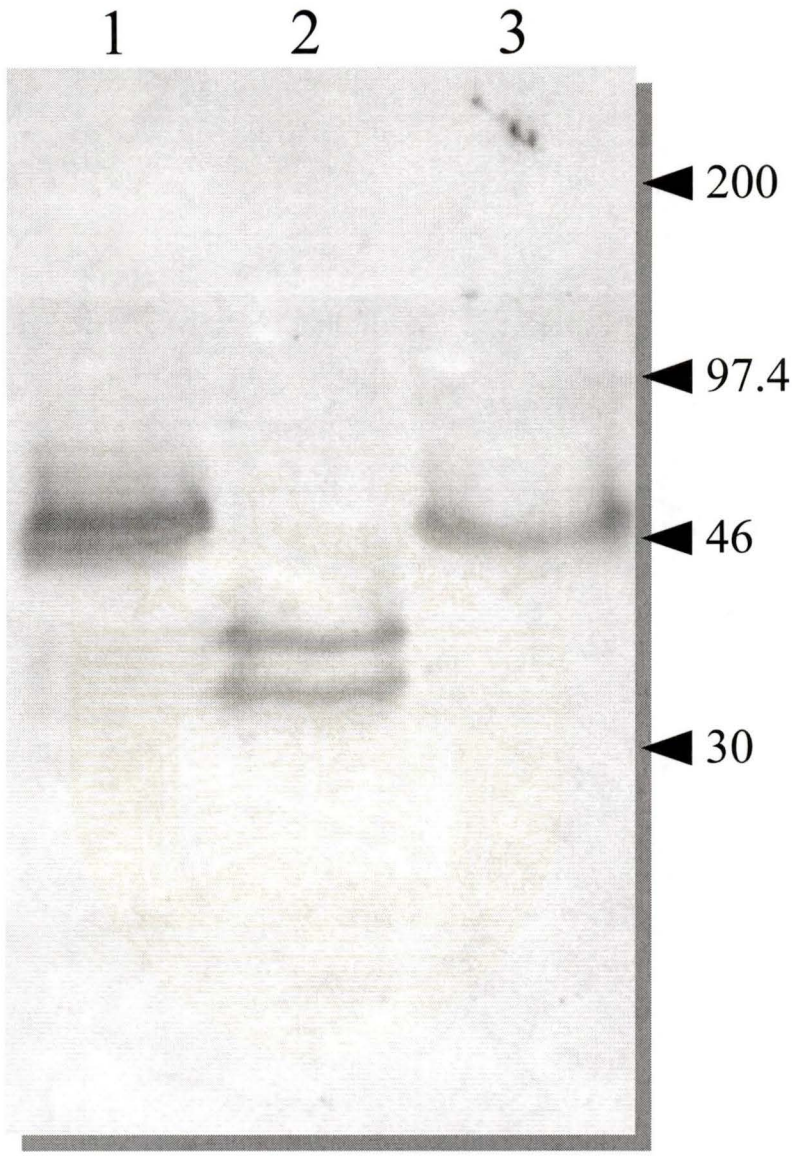

Figure 22: Western blot developed by the alkaline phosphatase method showing the effect of de-N glycosylation of rat erythrocyte membrane proteins on the 47 kDa ABP. Lane 1, untreated rat erythrocyte ghosts; lane 2, rat erythrocyte ghosts after treatment with PNGase F; lane 3, control rat erythrocytes. Molecular weights are given in kDa.

DISCUSSION

The work described in this thesis has revealed much about the identity and character of the aerolysin receptor on rat erythrocyte membranes. The receptor was first identified as a 47 kDa aerolysin binding species on a Western blot and subsequently shown to be a glycoprotein. A method was developed for its purification to near homogeneity and this enabled a partial N-terminal sequence to be derived. From these data it was discovered that the aerolysin receptor is related to a recently characterized family of proteins that includes several eukaryotic ADP-ribosyltransferases. The aerolysin receptor's identity as a novel member of this protein family was further demonstrated by determining that it, like its N-terminal homologues, is anchored to the cell membrane via a GPI anchor. Other techniques further characterized the receptor as described in this thesis.

Characterization of the Aerolysin Receptor

The results in Figure 3 show that the distribution of specific, high-affinity receptors for aerolysin is not restricted to rat erythrocytes. The pattern appears to fit well with what is known about the sensitivity of erythrocytes from different species to aerolysin (Bernheimer and Avigad, 1975; Howard and Buckley, 1982). Human erythrocytes, which are relatively insensitive to aerolysin, do not have a high affinity receptor species; rather, aerolysin seems to bind to these cells as a result of low affinity interactions with a number of membrane components. Glycophorin, one of the most

abundant glycoproteins on the erythrocyte surface, may act as the primary aerolysin-binding species on human erythrocytes but it does not bind the toxin with high affinity. Although human glycophorin was able to inhibit aerolysin mediated hemolysis of erythrocytes (Table 2) this does not appear to be the result of a specific aerolysin-receptor binding interaction, since neither the human nor the rat purified glycophorin preparations contained an aerolysin binding band as detected by the aerolysin sandwich Western blot method (Figure 6). Glycophorin's ability to inhibit aerolysin activity may result from its tendency to form micelles with negatively charged surfaces in solution to which aerolysin may bind and oligomerize (Springer *et al.*, 1966). The results in Figures 7 and 8 and Table 2 show that there is a correlation between the disappearance of intact glycophorin A as a result of protease digestion and the loss of all aerolysin inhibitory capacity. This seems to indicate that human glycophorin can only bind aerolysin, or can only form micelles when it is intact

The differences seen in the effects of proteases on the sensitivities of the human and rat erythrocytes to aerolysin also suggest that the mechanism of aerolysin binding is not identical in these species. Table 6 shows that after treatment with trypsin, the sensitivity of human erythrocytes to aerolysin increases slightly, whereas the sensitivity of rat erythrocytes decreased tenfold. In human erythrocytes chymotrypsin had the same effect as trypsin, causing an increase in aerolysin sensitivity but, in contrast, chymotrypsin treatment had no effect upon the sensitivity of rat erythrocytes. These results suggest that the aerolysin binds to rat erythrocytes via an interaction with a specific surface protein which is trypsin sensitive but chymotrypsin resistant. This is

confirmed by the blot result (Figure 19) for this experiment. Trypsin cleaves the 47 kDa band to yield a smaller aerolysin binding species (27 kDa), and over extended incubation (results not shown) eliminates the aerolysin binding species altogether. Chymotrypsin treatment had no effect upon the 47 kDa band. For human erythrocytes, the interpretation is not so clear. The increased sensitivity after pretreatment with either protease could suggest that the enzymes caused overall damage to the integrity of the membrane, hence making the erythrocytes more prone to lysis. If this were true, however, it might be expected that a similar effect would have been seen for the rat erythrocytes after chymotrypsin treatment. Alternatively, aerolysin mediated hemolysis of human erythrocytes may be enhanced after protease treatment because the removal of certain surface proteins improves access to the membrane for the aerolysin dimer or oligomer.

PNGase F de-N-glycosylation of the rat erythrocyte aerolysin receptor yields a smaller (32 kDa) aerolysin binding species, demonstrating that the receptor is an N-glycosylated protein. The N-linked glycans removed by this enzyme are not a part of the aerolysin binding epitope, since aerolysin is still able to bind to the deglycosylated form of the receptor (Figure 22). This may indicate that the epitope to which aerolysin binds is part of the 47 kDa receptor's amino acid sequence. However, the possibility that the protein also contains O-linked oligosaccharide chains has not been discounted, although this form of post-translational modification has not been reported for any of the receptor's homologues (i.e. RT6s, or GPI-anchored mono-ADP-RTs). There may also be additional N-linked glycan chains which were not removed by treatment with the PNGase F enzyme under the conditions used.

Both rat and mouse erythrocytes, which are amongst the most sensitive to aerolysin, exhibit a single high-affinity membrane receptor species for the toxin under the conditions used. Mouse T-cells (EL-4 in Figure 3) also have a high-affinity aerolysin receptor and it is likely that they share high aerolysin sensitivity with the rat erythrocytes. If so, the aerolysin secreted by *A. hydrophila* may be able to severely debilitate the mouse's immune response. It has been shown that as little as 0.06 μg of aerolysin is lethal for mice (Asao *et al.*, 1984). Whether the lethal dose pattern across species mirrors that of erythrocyte sensitivity remains to be revealed as the lethal dose in other species is unknown.

The variation in the apparent molecular mass of the aerolysin receptor between different cell types of the same species (31 kDa for the mouse T-cell, the same mass as mouse RT-6, and 50 kDa for the mouse erythrocyte) may be a result of differential post-translational processing of the same protein by each cell lines, for example, differences in the incorporation or composition of glycan side chains. Since both the rabbit muscle ADP-RT (Zolkiewska *et al.*, 1992) and the rat erythrocyte aerolysin receptor (Figure 22) seem to contain two N-glycosylation sites, it is possible that the mouse receptor could also contain multiple sites for N-linked glycosylation or other post-translational modification. The extent of modification could vary between mouse cell lines resulting in variation between the molecular masses of the receptor from different cell lines. Alternatively, the different receptor bands from mouse erythrocyte and mouse T-cells may represent two different proteins, although these proteins must presumably share some homology, since they are both recognized by aerolysin.

Possible Roles for the Aerolysin Receptor

The sequence of the amino terminal 25 amino acids of the receptor has significant homology with the N-terminal sequences of a family of proteins apparently involved in cell regulation and signalling via ADP-ribosylation of specific target proteins. This family includes GPI-anchored membrane proteins which have been characterized from avian erythrocytes (West and Moss, 1986), striated muscle (Okazaki *et al.*, 1994) and T-lymphocytes (Koch *et al.*, 1988; 1990).

The biochemical role of one of the members of this family of proteins is beginning to be elucidated. The rabbit striated muscle GPI-anchored ADP-RT is the protein with the highest sequence homology to the aerolysin receptor amino terminus. Closely related membrane bound ADP-RTs have been identified in the skeletal muscle of a number of species, including mouse and rat (McMahon *et al.*, 1993). The mouse protein has been demonstrated to ADP-ribosylate integrin $\alpha 7$ on the surface of skeletal muscle cells (Zolkiewska and Moss, 1993). Integrins are membrane spanning receptor proteins which regulate cell growth, migration and differentiation in response to extracellular stimuli (Hynes, 1992). Zolkiewska and Moss (1993) suggest that the ADP-ribosylation of integrin may alter its affinity for ligand, or otherwise regulate integrin function, thus influencing the ability of extracellular signals to alter muscle cell function.

More than 100 proteins are known to be attached to the external face of the plasma membrane by a GPI-anchor, yet the reason why they are anchored in this way is unclear. As outlined in the introduction, several hypotheses have been put forward to explain this form of membrane attachment for some of these proteins, but there is no

conclusive evidence to support any of them. Two of these proposals, that the GPI-anchoring may increase membrane motility of the proteins, or that it may be part of a novel internalization mechanism, are of particular interest with respect to an aerolysin receptor function. Firstly, if GPI-anchoring of proteins leads to high mobility in the bilayer (as proposed by Ishihara *et al.*, 1987 and Noda *et al.*, 1987), then such proteins would make ideal receptor candidates for a pore forming toxin, such as aerolysin, which must oligomerize in order to become active. The greater the membrane motility of the receptor, the greater the chance that bound aerolysin dimers will be brought together quickly, and be able to form functional oligomers. Secondly, it is proposed that some GPI-anchored proteins are involved in ligand internalization (Pu *et al.*, 1995). It has been demonstrated that they are able to gather and readily form clusters in particular regions on the cell surface (Stahl and Mueller, 1995; Pu *et al.*, 1995; Rothberg *et al.*, 1990). Such clustering could also promote the aggregation of aerolysin, and accelerate the formation of oligomers, assisting aerolysin mediated cytolysis.

It has been suggested that the ganglioside G_{M1}, which acts as a receptor for cholera toxin and the heat-labile enterotoxin of *E. coli*, may also have a role in the insertion of that toxin into the enterocyte membrane (Fishman, 1990). Fishman (1990) proposed that if CT bound in a multivalent fashion to G_{M1} the resultant cluster of gangliosides could produce a localized perturbation of the membrane, assisting protein insertion. Since the functional aerolysin multimer is heptameric it follows that the aerolysin oligomer could also exhibit multivalent binding of up to seven receptor molecules. Clustering of the GPI-anchored aerolysin receptor around an aerolysin

oligomer may have an important role in promoting both the insertion and pore formation processes.

Endogenous ADP-RTs are part of a cellular mechanism for the regulation of protein activity, and the potency of this form of regulation for key proteins such as EF-2 and actin makes them an important and exciting avenue for research. As is clear from the prevalence of ADP-RTs amongst the toxins of bacterial pathogens, any dysfunction or subversion of the normal cellular ADP-ribosylation cycle can be devastating. The binding, and possible regulation of one of these enzymes by aerolysin, not only on erythrocytes but also on nucleated cells (including T-cells) raises many questions. The possibility that by binding to its receptor aerolysin could, in turn, influence the ADP-RT activity could suggest a new mechanism of toxicity for aerolysin. If it can be demonstrated that the aerolysin receptor does possess endogenous ADP-RT activity, and that aerolysin binding can modulate that activity, then not only will this stimulate new avenues of research for aerolysin, and *Aeromonas* pathogenicity, but conversely, aerolysin could also prove a useful tool for the study of these eukaryotic proteins.

The Receptor-Binding Domain on Aerolysin

The region of aerolysin involved in the interaction with the target cell membrane receptor has been tentatively identified. Green and Buckley (1990) showed that a mutation of the histidine residue at position 332, in domain 2 of aerolysin, greatly reduced the binding of ^{125}I-labeled toxin to rat erythrocytes. They concluded that this residue must be at or near to the aerolysin receptor-binding site. By ^{125}I-labeling a

number of other potential aerolysin receptor-binding, site-directed mutants (i.e. those which were less hemolytically active than wild type, but were still able to oligomerize normally), other amino acid residues which might be a part of the binding site were investigated. Although the results obtained were inconclusive, they supported the theory of Green and Buckley that aerolysin may interact with its receptor through a region in domain 2 (see Figure 1). The construction of more site-directed aerolysin mutations in this region may enable the precise determination of which amino acids in the aerolysin sequence are responsible for receptor binding.

It is interesting that the receptor binding region is located in domain two of aerolysin. From the three dimensional structure of the aerolysin dimer (the normal *in vivo* form) this receptor binding region is in close proximity (Figure 1) to two regions found to be important for the oligomerization of aerolysin (Green and Buckley, 1990; van der Goot *et al.*, 1993). This suggests that receptor binding could have a role in stimulating oligomerization of the toxin to the membrane insertion competent form. It could be imagined that the interaction of the aerolysin monomer with the target cell receptor causes a shift in the aerolysin secondary structure, changing the orientation of those regions of the toxin which are located adjacent to the binding face. These regions contain several residues which are important for the oligomerization of the toxin (Figure 1), as has been demonstrated by site directed mutatagenesis (Green and Buckley 1990; Wilmsen *et al.*, 1991; van der Goot *et al.*, 1993). In addition, as mentioned previously, clustering of receptors carrying bound aerolysin may promote oligomerization. If the aerolysin bound to the receptor in a specific orientation such that those portions of the

toxin which are important for oligomerization were positioned so that they were brought into close contact during receptor clustering, then oligomerization would be greatly promoted.

Further evidence in support of the results presented in this thesis were obtained by a collaborating group in Austria. Gruber, Wilmsen and Aschauer were able to construct artificial bilayers and quantify aerolysin channel formation by measuring change in the current across them. These researchers demonstrated that the incorporation of rat erythrocyte membranes into their bilayers greatly increased the rate of aerolysin oligomer insertion. When fractions from the receptor purification protocol described here were tested using both the aerolysin Western blot protocol and this bilayer incorporation technique it was shown that the 47 kDa ABP peak fractions corresponded to those fractions with the ability to enhance the formation of oligomeric pores by aerolysin in a bilayer. The identification of the aerolysin receptor from rat erythrocytes as a GPI anchored protein was confirmed when this group demonstrated that rat erythrocyte membranes pretreated with PI-PLC no longer promoted aerolysin insertion when incorporated into artificial bilayers. In addition, they found that the penetration by aerolysin oligomers into bilayers which have the receptor incorporated into them is inhibited by treatment of the bilayers with PI-PLC.

The results described here demonstrate that the aerolysin receptor on the rat erythrocyte membrane is a GPI-anchored glycoprotein which migrates on SDS-PAGE

with an apparent molecular weight of 47 kDa. The protein is trypsin sensitive and bears at least two N-linked glycan chains, although these are not essential for the specific interaction with aerolysin. The N-terminus of the aerolysin receptor is homologous with the N-termini of several members of a family of GPI-linked cell surface proteins including the ADP-RT enzymes from striated muscle and the RT6 T-cell marker proteins

The discovery that the aerolysin receptor on rat erythrocyte membranes is related to a T-cell surface marker protein, and that a mouse T-cell line possesses a specific aerolysin receptor which is also GPI-anchored, has important implications for the understanding of aerolysin pathogenicity. By secreting aerolysin, *A. hydrophila* may be able to directly attack T-cells, a key component of the host's immune response, and an obstacle to invasion. This possibility is now being investigated.

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VITA

Surname: Cowell Given Names: Simon Piers

Place of Birth: Nantwich, Cheshire, Britain

Educational Institutions Attended:

University of Bath	1988-1992
University of Victoria	1993-1995

Degrees Awarded:

B.Sc. (Honours)	University of Bath	1992

Honours and Awards:

University of Victoria Fellowship	1993-1994

Publications

H.J. Gruber, H.U. Wilmsen, S. Cowell, H. Schindler, and J.T. Buckley. (1994) Partial Purification of the rat erythrocyte receptor for the channel forming toxin aerolysin and reconstitution into planar lipid bilayers. *Molecular Microbiology* **1478**:1093-1101

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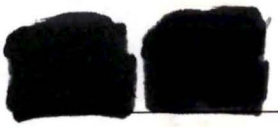
Author: 

Simon Piers Cowell

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