**Clostridium septicum** Alpha Toxin Uses Glycosylphosphatidylinositol-anchored Protein Receptors*


From the §Oral Infection and Immunity Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892, the ¶Department of Biochemistry and Microbiology, University of Victoria, British Columbia V8W 2Y2, Canada, the ‡Division of Cancer Biology, Department of Radiation Oncology, Emory University School of Medicine, Atlanta, Georgia 30335, the ‡Department of Microbiology and Immunology, BMSB-1011, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190, and the **Medicine Branch, Division of Clinical Sciences, NCI, National Institutes of Health, Bethesda, Maryland 20892

The alpha toxin produced by *Clostridium septicum* is a channel-forming protein that is an important contributor to the virulence of the organism. Chinese hamster ovary (CHO) cells are sensitive to low concentrations of the toxin, indicating that they contain toxin receptors. Using retroviral mutagenesis, a mutant CHO line (BAG15) was generated that is resistant to alpha toxin. FACS analysis showed that the mutant cells have lost the ability to bind the toxin, indicating that they lack an alpha toxin receptor. The mutant cells are also resistant to aerolysin, a channel-forming protein secreted by *Aeromonas* spp., which is structurally and functionally related to alpha toxin and which is known to bind to glycosylphosphatidylinositol (GPI)-anchored proteins, such as Thy-1. We obtained evidence that the BAG15 cells lack N-acetylg glucosaminy phosphatidylinositol deacetylase-L, needed for the second step in GPI anchor biosynthesis. Several lymphocyte cell lines lacking GPI-anchored proteins were also shown to be less sensitive to alpha toxin. On the other hand, the sensitivity of CHO cells to alpha toxin was increased when the cells were transfected with the GPI-anchored folate receptor. We conclude that alpha toxin, like aerolysin, binds to GPI-anchored protein receptors. Evidence is also presented that the two toxins bind to different subsets of GPI-anchored proteins.

Infection with *Clostridium septicum* is associated with a frequently fatal, nontraumatic gas gangrene (1). Distal myonecrosis due to *C. septicum* occurs mainly in individuals with certain predisposing conditions such as colon cancer, leukemia, neutropenia, and diabetes (2). Although this pathogen secretes a number of toxic proteins, including deoxyribonuclease and hyaluronidase, the lethal, cytolytic alpha toxin is implicated as a number of toxic proteins, including deoxyribonuclease and hyaluronidase, the lethal, cytolytic alpha toxin is implicated as the principal mediator of virulence (3). The *C. septicum* alpha toxin is a member of a group of pore-forming protein toxins, of which *Aeromonas hydrophila*

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† ‡ To whom correspondence should be addressed: Oral Infection and Immunity Branch, NIDCR, National Institutes of Health, Bldg. 30, Rm. 316, 30 Convent Dr., MSC 4350, Bethesda, MD 20892-4350. Tel.: 301-594-2865; Fax: 301-402-0396; E-mail: Leppla@nih.gov.

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1 The abbreviations used are: GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase; MTI, 3,4,5-trimethoxybenzyl-2,5-di-phenyltetrazolium bromide; PI-PLC, phosphatidylinositol-specific phospholipase C; FR, folate receptor; CHO, Chinese hamster ovary.

aerolysin is perhaps the best characterized member (4). In addition to their ability to form channels in target cell membranes, aerolysin and alpha toxin share several other properties, despite the fact that they are produced by bacteria widely separated in evolution. Both proteins are secreted from the bacteria as protoxins, and both are activated by proteolytic nicking near their C termini (5, 6). A number of proteases are capable of accomplishing activation, perhaps most notably the eukaryotic protease furin (7). Activation results in the formation of extremely stable oligomers that are believed to be the insertion-competent forms of the toxins (8). The crystal structure of proaerolysin has been solved (9). The protein consists of two lobes, a small lobe containing the first 80 amino acids of the protein and a larger lobe containing the rest of the protein. A number of studies have shown that the large lobe is involved in oligomerization of the toxin, as well as in activation (10, 11).

Remarkably, alpha toxin shares extensive sequence homology with the large lobe of aerolysin, accounting for some of the functional similarities between the toxins. Indeed, one of us has speculated that the two toxins have a common ancestor and that aerolysin obtained its smaller lobe by domain swapping (12). Recently a common fold has been observed in the small lobe of aerolysin and the S2 and S3 subunits of pertussis toxin (12). This fold is similar to the carbohydrate recognition domain of a number of proteins, implying it has a binding function in both toxins.

In the last several years, Howard and Buckley (13) showed that receptors that can bind aerolysin with high affinity can account for the sensitivity of some mammalian cells to the toxin, and also in the last several years, a number of aerolysin receptors have been identified on sensitive cells. These proteins appear to be unrelated except for one remarkable property: they are all attached to the cell surface by means of C-terminal glycosylphosphatidylinositol (GPI)1 anchors. Examples are Thy-1 (CD90), which is found in T-lymphocytes and brain (14), the neuronal surface molecule contactin (15), the 47-kDa erythrocyte aerolysin receptor (12, 16), and the variant surface glycoprotein of *Trypanosoma brucei* (15). Recent evidence indicates that the glycoyl portion of the receptor in the major binding determinant for aerolysin. Cell lines that lack the ability to make GPI anchors are much less sensitive to aerolysin (14), as are cells that have been treated with phosphatidyli-
inositol-specific phospholipase C (PI-PLC) (15), which can remove most GPI-anchored proteins (17).

Gordon et al. (7) explored the cellular requirements for alpha toxin sensitivity. One requirement is the presence of functional proteases to accomplish the conversion of the proform of the protein to the active toxin. Cells that lack furin, a surface protease that can correctly nick the protoxin, are lysed more slowly than those that express the protease (7). Another requirement for sensitivity is the ability of cells to localize alpha toxin on the cell surface, presumably through specific receptors. Until this time, no receptors have been identified for alpha toxin. Characterization of the receptors for other toxins has been aided by somatic cell mutagenesis methods (18, 19). In this communication, we describe isolation and characterization of an alpha toxin-resistant CHO cell that is unable to bind the toxin. We show that the mutant cells lack the ability to synthesize GPI anchors and provide other evidence that GPI-anchored proteins are receptors for alpha toxin.

**EXPERIMENTAL PROCEDURES**

**Materials**—*C. septicum* alpha toxin was produced in *Escherichia coli* as described previously (20). For biotinylation, 6 mg of alpha toxin in phosphate-buffered saline, pH 9.0, was reacted with 1 mg of sulfo-succinimidyl-biotin (Pierce, catalog no. 21335) for 2 h on ice. Toxicity tests showed that the biotinylated toxin was fully active. Aerolysin was purified from *A. hydrophila* as described previously (21). PI-PLC was purchased from Roche Molecular Biochemicals.

**Cell Lines**—Murine T lymphocyte cell lines AKR1 and EL4 and their derivatives, AKR1 (Thy-1+), and EL4 (Thy-1-), were generously provided by Dr. R. Hyman (Salk Institute). The murine T lymphocyte cell lines BW5147.3 and BW5147.3(Thy-1-) 10 were purchased from the ATCC. Lymphocyte cell lines were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g of glucose per liter, 10% (v/v) fetal bovine serum, and antibiotics (either 100 μg/ml streptomycin and 100 units/ml penicillin, or 50 μg/ml gentamicin). The CHO cell line transfected with the human folate receptor, referred to here as CHO FR-1, is the previously described CHO clone 2–8 (22). CHO cells were grown in α-minimal essential medium (Biofluids, Inc.) supplemented with 10% Fetal Clone II (HyClone, Logan, UT) and 50 μg/ml gentamicin.

CHO lec2 cells (23) were purchased from the ATCC. CHO lec2 cells were selected sequentially with 5 μg thigouagine and then 2 mM ouabain to obtain spontaneous mutants sensitive to alpha toxin. CHO lec2 cells were selected sequentially using a hypoxanthine/aminopterin/thymidine medium and resistant to ouabain. These marked lines are considered “universal hybridizers,” because they can be fused to unmarked cells for complementation analyses. In such fusions, both parents are killed by the combination of ouabain and hypoxanthine/aminopterin/thymidine medium, whereas fusion products survive. The resulting cloned cell line, CHO lec2 UH, was used as the parent for mutants that differ from CHO lec2 in toxin sensitivity, so these two lines were used interchangeably as controls in cytotoxicity comparisons with the mutant CHO cell line described below.

**Retroviral Mutagenesis of CHO lec2 UH Cells**—CHO lec2 UH cells were treated in the presence of 4 μg/ml Polybrene with four additions of 10 μg/ml Polybrene with four additions of 2 μg/ml streptogcin and 10 μg/ml native alpha toxin was added in precooled binding medium (minimum essential medium with Hanks’ salts) (Life Technologies, Inc., catalog no. 11570), containing a low NaICO3 concentration (4.5 mM), supplemented with 2 mM l-glutamic acid, 4 mM HEPES, pH 8.0, and 1% (v/v) bovine serum albumin). After a 1.5 h incubation at 4 °C, the plate was washed 5 times with chilled binding medium. Phycocerythrin-labeled streptavidin (1:1000, Molecular Probes, Inc) or binding medium (200 μl) was added, and the cells were incubated for 30 min at 4 °C, followed by extensive washing with binding medium. The cells were detached from the surface of the plates using 500 μl of a nonprotolytic, EDTA-based solution (Cello Dissocia-

**Cytotoxicity Assays with Adherent Cells (CHO)**—One day prior to the assay, cells were detached from flasks using trypsin-EDTA (Life Technologies, Inc.) and plated at 2 × 104 cells/ml in 96-well microtiter plates. The following day, toxin diluted in culture medium was added to the wells. Following 24 h, the plates were incubated for 1 h at 37 °C. Cell viability was assessed by the addition of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously (7). In assays in which CHO lec2 cells were treated with PI-PLC, adherent monolayers were incubated with 1 unit/ml PI-PLC for 60 min and then washed just prior to the addition of alpha toxin.

**Western Blotting Procedures**—Rat brain homogenate (1.0 ml) was treated with 400 milliliters of PI-PLC and centrifuged as described previously (24). The supernatant was dialyzed at 4 °C against the appropriate buffer before assay. The pellet was sonicated and centrifuged at 16,000 g for 20 min, and the pellets were resuspended and frozen in a solution of 10 mM HEPES, pH 7.5, 0.5 mM dithiothreitol, 0.1 mM tosyl-L-lysine chloromethylketone, 1 μg/ml leupeptin, and 10% glycerol. Protein was quantitated using the bicinchoninic acid assay (27). Biosynthesis of GPI intermediates from UDP-[6-H3]GlcNac (1 μCi) by microsomes (approximately 60 μg of protein) was measured in a reaction mixture of 50 mM HEPES, pH 7.5, 0.15 mM dithiothreitol, 0.15 μM native alpha toxin, 1 μg/ml leupeptin, 0.2 μg/ml tunicamycin, 1 mM ATP, and 1 mM EDTA (total volume of 300 μl). Deacetylation of [6-H3]GlcNac-Ac-PI (10,000 cpm), enzymatically prepared as described previously (26), was measured using the same conditions except that ATP and EDTA were omitted from the reaction mixture. GTP (1 mM) and CoA (1 μM) were included as indicated. After incubation for the indicated time at 37 °C, the reaction was stopped by the addition of 0.5 ml of H2O and 3 ml of chloroform/methanol (2:1 v/v) containing 0.1 N HCl. The radiolabeled GPI precursors were then extracted using the method of Bligh and Dyer (28) and analyzed by TLC as described previously (29).

**In Vitro Biosynthesis of GPI Intermediates**—The micromolar fraction was isolated from cells disrupted by nitrogen cavitation using differential centrifugation at 20,000 g (24). The resulting supernatant was applied to a gel filtration column (24). Fractions containing the GPI precursors were then extracted using the method of Bligh and Dyer (28).

**Glycosylation of GPI Intermediates**—To determine the site(s) of glycosylation of the GPI intermediates, fractions containing the GPI precursors were treated with 1 mg/ml trypsin at 37 °C for 1 h. The products were analyzed by SDS-PAGE.
alpha toxin and aerolysin.

CHO lec2 and CHO lec2 UH BAG15 cells were incubated with alpha toxin or aerolysin as described in the text for 1 h at 37 °C. MTT at 0.5 mg/ml was added, and the cells were incubated at 37 °C for an additional 1 h. The blue formazan crystals were dissolved, and cell viability was determined by measuring the absorbance at 590 nm. The data shown are for a single experiment that is representative of three or more assays.

RESULTS

Isolation of an Alpha Toxin-Resistant Cell Line by Retroviral Mutagenesis—We used retroviral insertional mutagenesis to identify genes required for the lysis of CHO cells by C. septicum alpha toxin. Recent work showed that CHO cells contain a cryptic receptor for Moloney murine leukemia virus (31). This receptor becomes functional when glycosylation is blocked, as can be achieved by treatment with tunicamycin or by mutation. Mutant CHO cell lines defective in various steps in glycosylation are available (23, 32). The CHO lec2 mutant cell line proved to be highly susceptible to the Moloney murine leukemia virus BAG (24) produced in the PA317 packaging cell line (ATCC CRL-9078). We selected spontaneous mutants of CHO lec2 having resistance to thioguanine and to ouabain so that any toxin-resistant mutants obtained could be analyzed by complementation analysis. The resulting universal hybridizer cell line was designated CHO lec2 UH. The introduction of the thioguanine and ouabain resistances did not affect sensitivity to any of the toxins used in this work.

Approximately 10^8 CHO lec2 UH cells were treated with the BAG retroviral vector. After allowing cell growth for expression of mutations, the cells were selected with 10 ng/ml alpha toxin, a dose previously found to kill >99% of the cells. Rare surviving cells were cloned and then compared with the parental line for sensitivity to several toxins. Resistant clones were obtained at a frequency of approximately 10^-7. One group of mutants displayed an intermediate level of resistance, with an EC_{50} (effective concentration causing 50% death) of about 200 ng/ml, as compared with an EC_{50} of 5 ng/ml for the CHO lec2 and CHO lec2 UH parents. This group has not been studied further.

Another group of mutants, obtained at about the same frequency, was completely resistant to the toxin. A mutant characteristic of this group, designated CHO lec2 UH BAG15, was unaffected by alpha toxin at concentrations of 1 μg/ml (2.2 × 10^-8 M), the highest concentration tested (Fig. 1, closed symbols). Comparable mutants, ones having intermediate and complete resistance to alpha toxin, were also obtained after chemical mutagenesis with ethylmethane sulfonate (data not shown). These chemically induced mutant cell lines have not been further characterized. Because it is expected that most loss of function mutations would be recessive, these results suggest that the loci being mutated by both the retroviral and chemical methods were already functionally hemizygous (i.e. haploid). CHO cells are unique in that they appear to be functionally hemizygous at many loci (33).

CHO lec2 UH BAG15 cells have lost the ability to bind alpha toxin. Cells grown to near confluence in 24-well plates were maintained at 4 °C and incubated with 1 μg/ml biotinylated alpha toxin (A) or 20 μg/ml native alpha toxin (B) for 1.5 h. The cells were washed, incubated with phycoerythrin-conjugated streptavidin for 0.5 h, washed again, and detached from the plates, and the fluorescence was analyzed by FACS. The solid line is the result with CHO lec2 cells, and the dotted line is the result with CHO lec2 UH BAG15 cells.

CHO lec2 UH BAG15 Cells Cannot Bind C. septicum Alpha Toxin—CHO lec2 UH BAG15 cells could be resistant to alpha toxin because they lack a surface receptor and are unable to bind the toxin. To investigate this possibility, the binding of alpha toxin to the parent and mutant cell lines was measured by FACScan analysis using biotinylated alpha toxin and a phycoerythrin-streptavidin conjugate. The parental cells were highly fluorescent, with a mean relative fluorescence intensity at least 10-fold above that of the mutant cells (Fig. 2A) and of controls using nonbiotinylated toxin (Fig. 2B). The low fluorescence of the mutant cells and of the controls is the intrinsic fluorescence of the cells, because it equaled that of cells not incubated with the phycoerythrin reagent (data not shown). The data show that the mutant cells have no detectable ability to bind alpha toxin.

CHO lec2 UH BAG15 Cells Are Also Insensitive to A. hydrophila Aerolysin—As stated earlier, alpha toxin is structurally and functionally related to the Aeromonas channel-forming protein. The nonbiotinylated form of this protein, termed aerolysin (35), is highly toxic to CHO cells (36). Like alpha toxin, aerolysin was toxic to the CHO parental line (ATCC CRL-9078). We selected spontaneous mutants of CHO lec2 having resistance to thioguanine and to ouabain so that any toxin-resistant mutants obtained could be analyzed by complementation analysis. The resulting universal hybridizer cell line was designated CHO lec2 UH. The introduction of the thioguanine and ouabain resistances did not affect sensitivity to any of the toxins used in this work.

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protein aerolysin, and it is likely the two toxins arose from a common ancestor (12, 20). It therefore seemed probable that the toxins may have similar receptors. Resistance of the CHO lec2 CHO lec2 BAG15 cells to aerolysin would imply that the two toxins bind to the same receptor. The CHO lec2 and CHO lec2 BAG15 cells were tested for sensitivity to aerolysin and alpha toxin in MTT assays (Fig. 1). The parental line, CHO lec2, was sensitive to both toxins, whereas CHO lec2 UH BAG15 cells were completely resistant to aerolysin at the concentration tested, as it was to alpha toxin.

CHO lec2 UH BAG15 Cells Are Deficient in the Second Step of GPI Anchor Biosynthesis—Based on the above results, it seemed likely that the mutant CHO cell line we produced would be deficient in synthesis of GPI-anchored proteins. The first three steps in GPI anchor biosynthesis are as follows.

\[
\text{UDP-GlcNAc + PI} \rightarrow \text{GlcNAc-PI} \rightarrow \text{GlcN-PI} \rightarrow \text{GlcN-PI(acyl)}
\]

(1) (2) (3)

Using different assay conditions to optimize each of these reactions (addition of GTP to stimulate the second reaction and GTP + CoA to stimulate the third reaction), we showed that CHO lec2 UH BAG15 cells were deficient in the second enzyme in GPI anchor biosynthesis, N-acetylglucosaminyl-phosphatidylinositol deacytelase (PIG-L). Microsomes prepared from parental and mutant cells were assayed for the ability to convert \(^3\text{H}-\text{UDP-GlcNAc}\) to the products of the three steps shown above (Fig. 3, lanes 1–6). All three products were synthesized normally by the parental cells (Fig. 3, lanes 1–3). The BAG15 microsomes synthesized GlcNAc-PI, the product of the first reaction, but neither of the later two products (Fig. 3, lanes 4–6), indicating that they were deficient in the second and possibly the third steps in GPI biosynthesis. To measure the second step in isolation, microsomes were incubated with \(^3\text{H}-\text{GlcNAc-PI}\). The parental cells, but not the mutant cells, were able to deacetylase this substrate to produce GlcN-PI (Fig. 3, lanes 7–10). These results show that the BAG15 mutant cells lack PIG-L. This enzyme has been shown to be missing in other CHO mutants defective in GPI biosynthesis (34, 35).

CHO Cells Treated with PI-PLC Are Less Sensitive to Alpha Toxin—One feature of the GPI-anchored proteins shown to be aerolysin receptors is that they can be removed from the cell surface by treatment with PI-PLC, and it has been established that cell sensitivity to aerolysin is greatly reduced by this treatment (15). Treatment of CHO lec2 cells with PI-PLC reduced their sensitivity to alpha toxin by about 10-fold (Fig. 4), providing more evidence that the clostridial toxin binds to a surface GPI-anchored protein.

Cells Lines Lacking GPI-anchored Proteins Are Less Sensitive to Alpha Toxin—A number of cell lines are known to lack specific enzymes required for the synthesis of GPI anchors and as a result cannot retain proteins that are normally anchored in this way on their surfaces (36, 37). Several of these cell lines, including the mouse lymphocyte mutant cell line EL4 (Thy-1+), have been shown to be resistant to aerolysin, and this has been used as more evidence that aerolysin binds to GPI-anchored proteins (14). The results in Fig. 5 illustrate that these cells are also resistant to alpha toxin, whereas the parental EL4 cell line is sensitive.

Alpha Toxin Binds Specifically to GPI-anchored Proteins—Using a sandwich Western blotting procedure, we previously identified several proteins in brain and erythrocytes that bind aerolysin (14–16, 30). These toxin-binding proteins are easily observed in Fig. 6A. Supernatants from PI-PLC-treated brain extracts contain two major GPI-anchored proteins, contactin, which migrates at ~110 kDa, and Thy-1, at ~35 kDa (Fig. 6A, lane 1). Three faint bands migrating between contactin and Thy-1 are also observed. A single aerolysin-binding protein of 47 kDa, which we have also shown is GPI-anchored (30), can be identified in erythrocytes (Fig. 6A, lane 2). We screened the same samples of brain and erythrocytes with alpha toxin, using the same Western blotting procedure. The results in Fig. 6B show that alpha toxin binds to a band corresponding to contactin in brain as well as to the three minor GPI-anchored proteins that bound aerolysin. Surprisingly, the blotting procedure with alpha toxin did not detect Thy-1, despite the fact that it was the strongest band detected with aerolysin and that it is probably the major GPI-anchored protein in brain. The alpha toxin blotting procedure also did not detect the 47-kDa aerolysin-binding protein of erythrocytes.

GPI-anchored Proteins Other Than Thy-1 Can Serve as Alpha Toxin Receptors in Mouse T-Lymphocytes—Because alpha toxin does not appear to bind to Thy-1, we asked whether cells lacking only this one GPI-anchored protein would be sensitive to the toxin. The mutant T-lymphocyte cell line AKR1 (Thy-
d) is unable to make Thy-1 due to mutation of the structural gene, but it retains the ability to make other GPI-anchored proteins. The results in Fig. 7 show that this cell line is as sensitive to alpha toxin as the parental cell line, AKR1 (Thy-1). However, the cells became about 10-fold less sensitive to alpha toxin after treatment with PI-PLC. These data indicate that there is at least one GPI-anchored protein other than Thy-1 in T-lymphocytes that can act as a receptor for alpha toxin. We have drawn a similar conclusion with aerolysin.

Expression of Human Folate Receptor Increases the Sensitivity of CHO Cells to Alpha Toxin—The above results suggest that alpha toxin, like aerolysin, can bind to several different GPI-anchored proteins. We reasoned that the sensitivity of CHO cells to the toxin might be increased if the cells were transfected with a gene encoding a GPI-anchored protein. We chose to use the folate receptor gene, which is not expressed in wild type CHO cells (22). A CHO line transfected with the folate receptor gene, CHO FR+, was 3–5-fold more sensitive to alpha toxin than the parental CHO cell line (Fig. 8). When extracts of these cells were examined by probing blots with biotinylated alpha toxin and streptavidin-HRP, a band was detected having the size expected for the recombinant folate receptor, 38 kDa (Fig. 9, lane 4). Four additional bands detected in all the cell extracts, including the PIG-L-deficient BAG15 cells, correspond in size to the known, endogenous, intracellular, biotin-containing carboxylases (38). Apparently these proteins are binding the streptavidin-HRP independent of the presence of alpha toxin. Interestingly, no alpha toxin binding bands were detected in the CHO lec2 cells that were absent in the BAG15 cells. Because our evidence that the wild
type cells contain a GPI-anchored alpha toxin binding protein is compelling (Figs. 1, 2, 4, and 9), this suggests that the Western blotting procedure used here does not detect all alpha toxin binding proteins. We have drawn a similar conclusion with aerolysin (15).

**Solubilized Folate Receptor Is Recognized by Alpha Toxin**—Because treatment of CHO cells with PI-PLC reduces their sensitivity to alpha toxin (Fig. 4), it was predicted that PI-PLC treatment of the CHO FR+ cells would release the folate receptor. Western blot analysis showed that this was the case and that the folate receptor released into the supernatant by PI-PLC treatment retained the ability to be recognized by alpha toxin (Fig. 10). This result is consistent with the recognition by aerolysin of contactin and Thy-1 released from rat brain homogenates by PI-PLC treatment (Fig. 6). Clearly, the structures recognized by alpha toxin and aerolysin do not include the diglyceride moiety of the GPI anchor, which is cleaved off by PI-PLC and remains in the cell membrane.

**Aerolysin Does Not Bind to the GPI-anchored Folate Receptor in CHO Cells**—The above results provide strong evidence that alpha toxin can bind to the folate receptor when it is expressed in CHO cells. However, the results in Fig. 8 also show that expression of the folate receptor did not increase sensitivity to aerolysin. Consistent with this were the results of a Western blot analysis involving incubation with native toxins followed sequentially with the corresponding anti-toxin antibody and a secondary, HRP-conjugated antibody (Fig. 11). The folate receptor was much more strongly recognized by alpha toxin (Fig. 11A) than by aerolysin (Fig. 11B).

**GPI-deficient Cells That Are Completely Insensitive to Alpha Toxin Retain Some Sensitivity to Aerolysin**—Further evidence that the two toxins have different receptors is provided from data obtained using other GPI anchor-deficient cell lines. The wild type cell line BW5147.3 is sensitive to both toxins. A mutant of this cell line lacking GPI-anchored proteins, BW5147.3(Thy-1-1)-e).10, is completely resistant to alpha toxin (Fig. 12), but retains some limited sensitivity to aerolysin. A difference in action of the two toxins was also seen in Fig. 5, but not in Fig. 1. The fact that all GPI anchor-deficient cells are insensitive to alpha toxin suggests that GPI-anchored proteins are the sole means of attachment of this toxin to cells. The mutant cells retain some sensitivity to aerolysin because this *Aeromonas* toxin contains the carbohydrate-binding small lobe that mediates binding to membrane proteins, such as glycoporphin, that are not GPI-anchored (39).2

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2 C. R. Mackenzie and J. T. Buckley, manuscript in preparation.

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cause it is difficult to achieve release of more than 90% of GPI-anchored proteins from the cell surface by PI-PLC treatment (17) and because new GPI-anchored proteins are delivered to the cell surface during the exposure to toxin.

Several T lymphocyte cell lines that are unable to make GPI-anchored proteins are known to be resistant to aerolysin, and this gave us a clue to the mutation in the BAG15 clone. We were then able to show that the cells are deficient in the second enzyme in the pathway, PIG-L. Comparable mutates of other cell lines have been described previously (35, 40).

Although the anchors of all GPI-anchored proteins have the same core structure, there are species and cell type variations in the sugars and phosphoethanolamines that modify the core (37). Our results show that the relative affinities of aerolysin and alpha toxin for individual proteins with these anchors differ. This was especially striking with two proteins, Thy-1, which is bound much more strongly by aerolysin than by alpha toxin, and the folate receptor, which is bound more strongly by alpha toxin. Thus there must be a difference in the structures of the two toxins that affects the specificity of their interactions with different GPI-anchored proteins. The most obvious difference is the presence of the small lobe in aerolysin (12). This lobe contains a fold that is similar to the carbohydrate-binding domains of other proteins, and we have recent evidence that it facilitates aerolysin binding to GPI-anchored proteins as well as to glycoproteins that are not GPI-anchored. The presence of the small lobe in aerolysin accounts for the fact that certain GPI anchor-deficient cells retain some sensitivity to the Aeromonas toxin although they are completely resistant to alpha toxin. The ability of aerolysin to act on GPI anchor-deficient cells might, for example, involve a low affinity interaction of the small lobe with cell surface glycoproteins or glycolipids. The absence of certain glycosylations in the CHO lec2 parent of the BAG15 mutant would then explain the complete resistance of the BAG15 mutant to aerolysin (Fig. 1). The alpha toxin, lacking a carbohydrate recognition region, would not have residual affinity for GPI anchor-deficient cells, even those fully proficient in glycosylation.

The steps in GPI anchor biosynthesis are biochemically well defined. Nevertheless, it may be useful to exploit the powerful selective ability of alpha toxin and aerolysin to produce additional mutates defective in GPI synthesis. It may be particularly useful to produce such mutants in CHO cells, because the hearty nature and rapid growth of these cells make them a convenient model system.

The strong selective action of aerolysin and alpha toxin on cells containing GPI-anchored proteins suggests that they could be useful reagents in genetic manipulation of cells. For example, to achieve site-specific integration of an exogenous gene by homologous recombination, one could design targeting vectors containing flanking regions of a gene such as PIG-L that encodes an enzyme essential to GPI anchor synthesis. Successful homologous integration would disrupt the gene, allowing selection of the rare integrants by treatment with the toxins. Alternatively, in mutant cells lacking an enzyme such as PIG-L, an expression vector containing the PIG-L gene could provide a positive selection, with transfected cells being selected by FACS or magnetic cell sorting.

Finally, we anticipate that the recognition that two related toxins bind differently to GPI-anchored proteins will aid attempts to understand the structural basis for interaction of these toxins with receptors. It will be especially useful to measure the affinities of the two toxins for the GPI anchor and then to study the effects of structural variations in the GPI core structure and its accessory modifications. One can anticipate that solving the structure of the toxin complexed with a GPI anchor will be particularly informative and can be achieved by using the known structure of aerolysin (9).

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Clostridium septicum Alpha Toxin Uses Glycosylphosphatidylinositol-anchored Protein Receptors

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