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Use of *Clostridium septicum* Alpha Toxins for Isolation of Various Glycosylphosphatidylinositol-Deficient Cells

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In eukaryotic cells, various proteins are anchored to the plasma membrane through glycosylphosphatidylinositol (GPI). To study the biosynthetic pathways and modifications of GPI, various mutant cells have been isolated from the cells of Chinese hamster ovaries (CHO) supplemented with several exogenous genes involved in GPI biosynthesis using aerolysin, a toxin secreted from gram-negative bacterium *Aeromonas hydrophila*. Alpha toxin from Gram-positive bacterium *Clostridium septicum* is homologous to large lobes (LL) of aerolysin, binds GPI-anchored proteins and possesses a cell-destroying mechanism similar to aerolysin. Here, to determine whether alpha toxins can be used as an isolation tool of GPI-mutants, like aerolysin, CHO cells stably transfected with several exogenous genes involved in GPI biosynthesis were chemically mutagenized and cultured in a medium containing alpha toxins. We isolated six mutants highly resistant to alpha toxins and deficient in GPI biosynthesis. By genetic complementation, we determined that one mutant cell was defective of the second subunit of dolichol phosphate mannose synthase (*DPM2*) and other five cells were of a putative catalytic subunit of inositol acyltransferase (*PIG-W*). Therefore, *C. septicum* alpha toxins are a useful screening probe for the isolation of various GPI-mutant cells.

Key words: glycosylphosphatidylinositol, *Aeromonas hydrophila*, aerolysin, *Clostridium septicum*, alpha toxins

Glycosylphosphatidylinositol (GPI) is a complex glycophospholipid that acts as a signal for protein sorting in epithelial cells, for signal transduction, for immune responses and for pathology of infectious diseases (Ikezawa, 2002). Biosynthetic pathways of GPI are mediated by the sequential additions of sugars and other components to phosphatidylinositol (PI). Finally, GPI preassembled in the ER is transferred *en bloc* to proteins bearing the GPI attachment signals at the C-terminus (Ferguson, 1999; Gowda and Davidson, 1999; Eisenhaber *et al.*, 2003).

In yeast and other unicellular parasites, e.g. *Trypanosoma* and *Leishmania*, GPI is essential for the integrity and growth of the outer cell membrane (Nagamune *et al.*, 2000). However, in mammals, GPI is not essential at the cell level, although its deficiency causes the embryonic lethality and improper development of the skin in mice (Tarutani *et al.*, 1997). In humans, a lack of GPI biosynthesis causes an acquired hematopoietic stem cell disorder, paroxysmal nocturnal hemoglobinuria (PNH), in blood cell subpopulations (Takeda *et al.*, 1993).

The Gram-positive bacterium *Clostridium septicum* secretes the hemolysin alpha toxins, forming a channel on the host cell membrane (Ballard *et al.*, 1992). The receptors for alpha toxins binding are identified to be GPI-anchored proteins (Diep *et al.*, 1998; Gordon *et al.*, 1999). The amino acid sequence and functional mechanism of alpha toxins are homologous to those of aerolysin and enterolobin of the Gram-negative bacterium *Aeromonas hydrophila* and the plant *Enterolobium contortisiliquum*, respectively (Sousa *et al.*, 1994; Ballard *et al.*, 1995; Fivaz *et al.*, 2001). Consequently, these three toxins constitute the aerolysin channel-forming toxin family. Upon binding to GPI-anchored proteins, the C-terminal peptide of these toxins is cleaved by furin-like proteases on the outer cell membrane (van der Goot *et al.*, 1994; Sellman and Tweten, 1997; Abrami *et al.*, 2000). This C-terminal cleavage activates the toxins because the cleavage of the C-terminal peptide exposes their hydrophobic domains, which are necessary for subsequently inducing their oligomerization. The aggregated oligomeric toxins then are inserted into the cell membrane and form pores. Those pores permit the free passage of low weight ions and sugars, and, eventually, destroy the cell (Fivaz *et al.*, 2001). Therefore, amino acid residues of alpha toxins conserved to aerolysin are functionally important. We recently reported mutants of alpha toxins that were able to bind GPI but lost cytotoxicity (Shin *et al.*, 2004).

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GPI is synthesized step-by-step by sequential addition and modification of sugars and some molecules, which are derived by enzymes consisting of at least one component (Eisenhaber *et al.*, 2003). However, it is difficult to directly characterize such enzymes because most of them are membrane proteins in endoplasmic reticulum (ER). Therefore, the characterization of genes or their products has usually been done in mutant cells. Over twenty mutant cells defective of genes related to GPI biosynthesis and their responsible genes have been cloned and characterized (Kinoshita and Inoue, 2000). At first, the method to isolate GPI-deficient cells by complement lysis or cell sorting after staining the cells with antibodies against specific GPI-anchored proteins have been used (Nakamura *et al.*, 1997). However, this method required expensive instruments, such as cell sorter and reagents such as complements or antibodies. In this point, toxins recognizing GPI-anchored proteins are valuable for GPI-mutant screening. Using aerolysin and *C. septicum* alpha toxins to the cells of Chinese hamster ovaries (CHO), class L GPI-mutant cells were isolated, which were defective of PIG-L deacetylase (Gordon *et al.*, 1999). When CHO cells stably transfected with PIG-L and several cDNA related to GPI biosynthesis were used in aerolysin screening, novel mutant cells were able to be isolated. However, the mutant cells defective of N-glycosylation modification were also isolated in aerolysin screening. (Hong *et al.*, 2002) The reason is that aerolysin consists of two discrete globular structures, namely, the N-terminal small lobe (SL) and the C-terminal large lobe (LL) (Parker *et al.*, 1994). The SL and LL domains recognize the N-glycan and GPI moiety of GPI-anchored proteins, respectively (Hong *et al.*, 2002). Unlike aerolysin, *C. septicum* alpha toxins only possess the LL structure (Ballard *et al.*, 1995). Thus, it appears that alpha toxins do not require the N-glycan moiety to bind to its receptor on the outer cell membrane of the host (Hong *et al.*, 2002; Shin *et al.*, 2004).

In this paper, we report that *C. septicum* alpha toxins as well as aerolysin could be used as a selecting agent to isolate various GPI-mutant cells from a colony of CHO cells.

Materials and Methods

Cell culture and Plasmids

D9(wt) (Murakami *et al.*, 2003; Kang *et al.*, 2005) and mutant CHO cells were cultured in the F-12 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C.

All cDNA used in this work were constructed in a mammalian expression vector, pME (gifts from Dr. Taroh Kinoshita, Osaka University, Japan).

Isolation of mutant cells resistant to C. septicum alpha toxins

D9(wt) cells were CHO cells stably transfected with *CD59, DAF, PIG-L, DPM2, SL15, PIG-A, PIG-F, PIG-O, PIG-U,* and *GnT1* (Murakami *et al.*, 2003; Kang *et al.*, 2005). In these cells, CD59 and DAF were GPI-anchored proteins used as markers. Chemical mutagenesis was done by ethyl-methanesulfonate (EMS) as described (Hong *et al.*, 2003) and mutangenized cells were treated with 1 nM GST-tagged alpha toxins (GST-AT) for 9 h (Shin *et al.*, 2004). After GST-AT treatment, the plates were cultured in a fresh medium to allow for the formation of colonies of surviving cells and subjected to further treatment of GST-AT. The cells were then cloned by the limiting dilution and analyzed by FACS analysis after staining with anti-CD59 and anti-DAF antibodies.

Cell viability assay

The viability of cells treated with alpha toxins was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously (Quinn *et al.*, 1991). The cells (2×10^4/well) were cultured at 37°C for 1 day in 96-well microtiter plates for attachment. The next day, the cells were incubated with 100 μl of GST-AT diluted in culture medium at 37°C for 3 h, and then MTT was added to provide a final concentration of 0.5 mg/ml. After an additional incubation for 1 h and removal of media, accumulated blue tetrazolium crystal was dissolved in 100 μl SDS in acidic isopropanol. Absorption at 570 and 630 nm was measured by microplate reader (Molecular Devices, USA) to determine the amount of blue tetrazolium salt. The percentage of viability was calculated as:

$$100\times[\text{toxin-treated } (A_{570} - A_{630}) - \text{background } (A_{570} - A_{630})] / [\text{non-toxin-treated } (A_{570} - A_{630}) - \text{background } (A_{570} - A_{630})].$$

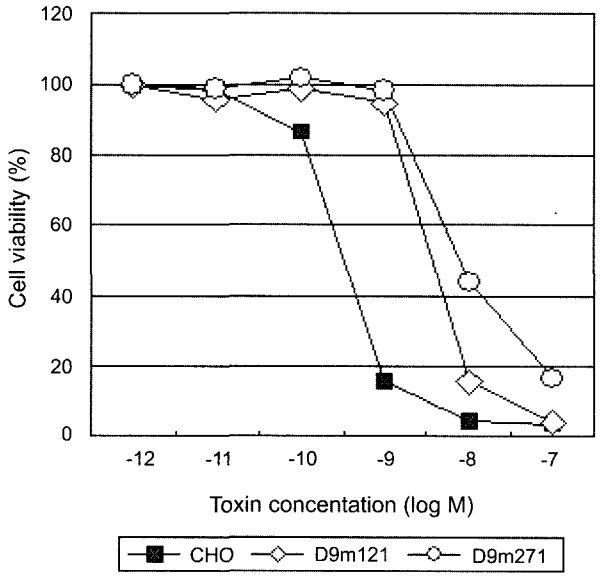

Fig. 1. Resistance of D9m121 and D9m271 cell against *C. septicum* alpha toxins. CHO, D9m121, and D9m271 cells were treated with various concentrations of GST-AT. After 3 h at 37°C cell viability was measured by MTT.

Transfection

Transfection of GPI-deficient mutant cells was performed by the Lipofectamine 2000 (Invitrogen, USA). The cells (5×10^5) were cultured on a 12-well plate for 1 day and washed with Opti-MEM I (Invitrogen, USA), and incubated in the same media with 3% FBS. Plasmid DNAs (2 μg) and Lipofectamine 2000 (4 μl) were mixed in 100 μl Opti-MEM I, respectively, and stored at room temperature for 5 min. Then, DNA and Lipofectamine 2000 were mixed and incubated for 20 min at room temperature, and added to the cells. After culturing for 3 h at 37°C, the media were changed with fresh F-12 media containing only 10% FBS and incubated for 24 h at 37°C.

Fluorescent activated cell sorting (FACS) analysis

To see the expression levels of CD59 and DAF, the cells were stained with anti-CD59 antibody (5H8) plus FITC-conjugated anti-mouse IgG antibody and biotinylated

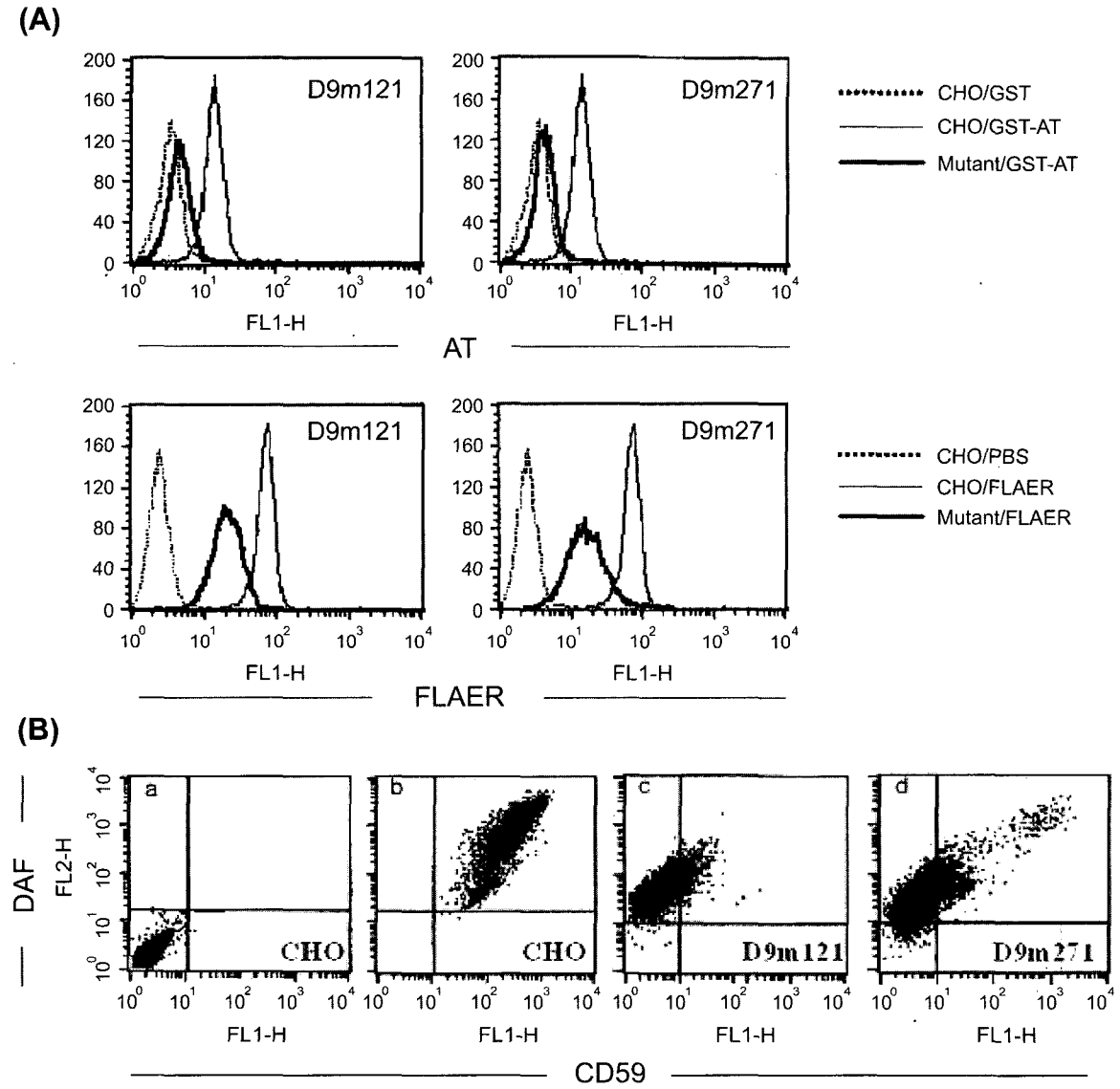

Fig. 2. D9m121 and D9m271 cells did not express GPI-anchored protein on the outer cell membrane.

A. Binding of *C. septicum* alpha toxins and aerolysin to D9m121 and D9m271 cells. To see alpha toxins binding, mutant cells were treated with 50 nM of GST-AT (*upper panels*). After washing, the cells were sequentially stained with mouse anti-GST antibody and FITC-conjugated anti-mouse IgG antibody and analyzed by FACS. As a control, D9(wt) CHO cells were treated with GST or GAT-AT. To see aerolysin binding, mutant cells were stained with 50 nM of FLAER (*lower panels*). As a control, D9(wt) CHO cells were stained with buffer or FLAER. Numbers at y-axis represent cell counts.

B. Expression profile of GPI-anchored proteins on D9(wt) CHO, D9m121, and D9m271 cells. The cells were dually stained with anti-CD59 and anti-DAF antibodies and analyzed by FACS. a, D9(wt) CHO cells stained with isotype non-relevant antibodies; b, D9(wt) CHO cells stained with anti-CD59 and anti-DAF antibodies; c, D9m121 cells stained with anti-CD59 and anti-DAF antibodies; d, D9m271 cells stained with anti-CD59 and anti-DAF antibodies.

anti-DAF antibody (IA10) plus PE-conjugated streptavidin (Biomeda, USA). The cells were also stained with 50 nM Alexa488-conjugated proaerolysin (FLAER) (Protox, Canada) or GST-AT for 15 min on ice followed by further staining with mouse monoclonal anti-GST antibody (Sigma, USA) and FITC-conjugated anti-mouse IgG antibody (Biomeda, USA) The stained cells were analyzed by FACScalibur (Becton Dickinson, USA).

Results and Discussion

Isolation of CHO mutant cells resistant to C. septicum alpha toxins

The D9(wt) cell was used for the isolation of class V and class W mutant cells in GPI biosynthesis using aerolysin (Murakami *et al.*, 2003; Kang *et al.*, 2005). These cells were stably transfected with the cDNA of ten genes involved in GPI-anchored marker proteins, GPI biosynthetic pathway and N-glycosylation; *CD59, DAF, PIG-L, DPM2, SL15, PIG-A, PIG-O, PIG-U, PIG-F,* and *GnT1*. After EMS mutagenesis, cells were treated with alpha toxins for 9 h. Finally, we selected 6 cells that were highly resistant to alpha toxins and further characterized them.

At first, we examined cell viability of the six mutants using MTT assay after alpha toxin treatment for 3 h. 10^{-9} M alpha toxins killed 80% wild-type D9(wt) cells but not affect cell viability of all mutant cells. Toxin concentration above 10^{-8} M was required to kill mutant cells to the similar level of wild type cells (Fig. 1). It indicated that mutant cells were at least 10-fold highly resistant to alpha toxin. Next, we tested whether mutant cells possessed the receptors for alpha toxins on the outer cell membrane. To do this, we treated the cells with GST-tagged alpha toxins (GST-AT) and analyzed in FACS. Compared to wild-type D9(wt) cells, D9m121 and D9m271 mutants showed almost no binding against alpha toxins (Fig. 2A, *upper panels*). It indicated that the resistance of mutant cells against alpha toxins was caused by the low number of receptors on the outer cell membrane, that is, GPI-anchored proteins.

If mutant cells were deficient of GPI-anchored proteins, the binding against aerolysin also would be decreased because aerolysin uses the same GPI-anchored proteins as a receptor. Therefore, we stained the mutant cells with fluorescent-tagged aerolysin (FLAER) (Fig. 2A, *lower panels*). The mutant cells showed decreased binding of aerolysin compared to D9(wt) cells. Nevertheless, aerolysin still bound the mutant cells significantly, compared to non-stained cells. It indicates that mutant cells would be mutated only in GPI biosynthesis but not in N-glycosylation since SL domain of aerolysin recognized N-glycan on the outer cell membrane (Hong *et al.*, 2002; Shin *et al.*, 2004).

Finally, to investigate the expression of GPI-anchored proteins on the outer cell membrane of mutants, we stained cells with antibodies against GPI-anchored proteins; DAF and CD59 (Fig. 2B). Wild-type D9(wt) cells showed high expression of GPI-anchored proteins. D9m121 cells showed over 100-fold expression decrease of CD59 and the 10-fold decrease of DAF. Independent clones, D9m122, D9m311, D9m422 and D9m433 showed the same phenotypes with D9m121 cells in binding with alpha toxins, aerolysin and the staining with antibodies against CD59 and DAF (data not shown). The expression of CD59 and DAF was mostly deficient in D9m271 cells like D9m121 cells, but D9m271 cells showed small population of CD59 and DAF expression. It means that these cells possess a little remained activity of GPI biosynthesis.

Taken together, it indicated that all mutant cells resistant to alpha toxins were deficient of GPI-anchored proteins on the outer cell membrane.

CHO mutant cells resistant to Clostridium alpha toxins were defective in GPI biosynthesis

To know at which step mutant cells were defective, we transiently transfected all known cDNA involved in GPI biosynthesis into the cells. In D9m121 cell, the recovery of GPI-anchored proteins was detected on the outer cell membrane, when the cells were transfected with only *PIG-W* cDNA encoding a catalytic subunit of inositol acyltransferase(Murakami *et al.*, 2003) (Fig. 3). Also, D9m122, D9m311, D9m422 and D9m433 cells transfected with *PIG-W* cDNA showed the recovery of GPI-anchored proteins (data not shown). It indicated that these five cells belonged in the class W mutants, defective of *PIG-W* gene.

The expression of GPI-anchored proteins in D9m271 cells was recovered by the transfection with DPM (dolichol phosphate mannose) synthase genes. In mammalian and *Schizosaccharomyces pombe* but not *Saccharomyces cerevisiae*, this enzyme is consist of three components: 1) DPM1 with a catalytic domain; 2) DPM2 with an enzyme

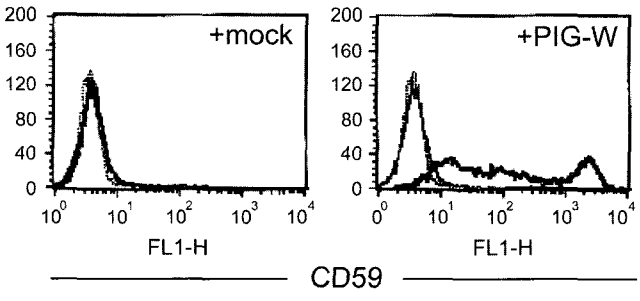

Fig. 3. Defect of *PIG-W* gene in D9m121 cells. D9m121 cells were transiently transfected with mock and *PIG-W* cDNAs. After 2 days, the cells were detached by trypsin and stained with anti CD59 antibody. Dotted lines, non-stained D9m121 cells; thin lines, D9m121 cells stained with anti-CD59 antibody; thick lines, transfected D9m121 cells stained with anti-CD59 antibody. Numbers at y-axis represent cell counts.

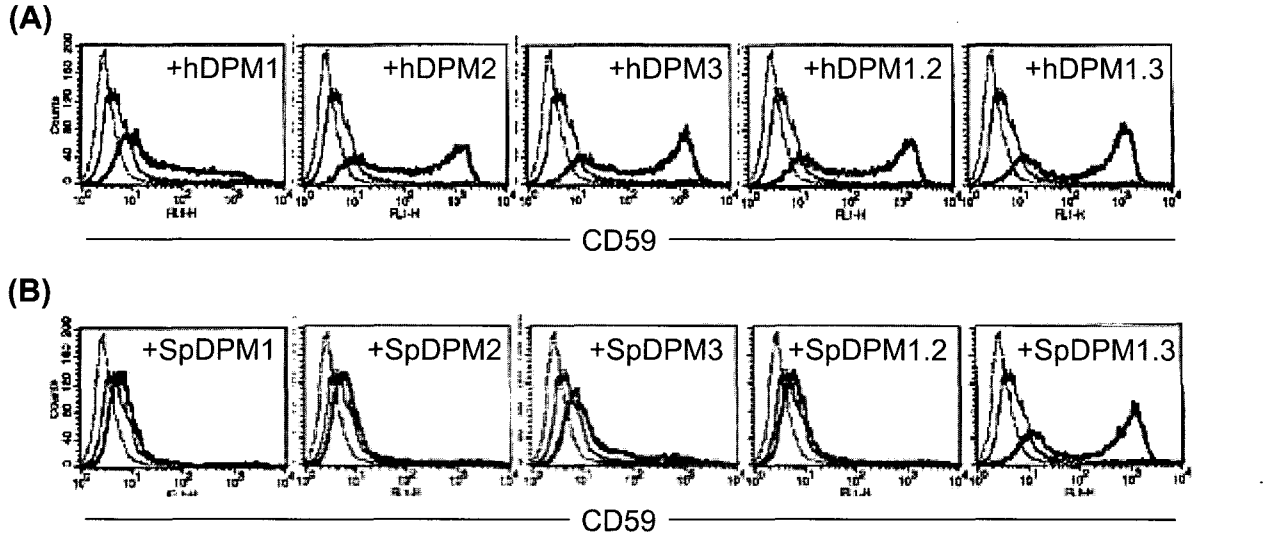

Fig. 4. Genetic complementation of D9m271 cells with dolichol-phosphate mannose (DPM) synthase genes. D9m271 cells were transiently transfected with human (A) and *Schizosaccharomyces pombe* (B) *DPM1*, *DPM2*, *DPM3*, or their combinations. After 2-day culture, the cells were stained with anti-CD59 antibody and subjected to FACS analysis. Dotted lines, non-stained D9m271 cells; thin lines, D9m271 cells stained with anti-CD59 antibody; thick lines, transfected D9m271 cells stained with anti-CD59 antibody.

stablizer and ER localizing function; and 3) DPM3 with an unknown function (Maeda *et al.*, 1998; Maeda *et al.*, 2000). In our experiment, *DPM1* partially recovered the GPI-anchored protein (Fig. 4A). It indicated that this mutant was defective of *DPM2* or *DPM3*. Cells with *DPM2* or *DPM3* expressed the GPI-anchored proteins to wild-type level. This phenotype is similar to Lec15 cells, which is defective of *DPM2* (Ware and Lehrman, 1998; Maeda *et al.*, 2000). To clarify whether D9m271 possessed the same phenotype as Lec15 cells, we transiently transfected *S. pombe* homologues of *DPM1*, *DPM2* and *DPM3* genes into D9m271 cells (Fig. 4B). Single transfection with *SpDPM3* but not *SpDPM1* or *SpDPM2* into D9m271 cells showed a partial recovery of GPI-anchored protein. However, the cells transfected with both *SpDPM1* and *SpDPM3* showed much more expression of GPI-anchored proteins than one transfected with only *SpDPM3*. This phenotype in transfection by *S. pombe* DPM synthase genes was the same as Lec15 cells. It is not possible that D9m271 cells are defective of *DPM3*, because the *DPM3* mutant cells could recover the GPI-anchored expression by *DPM3* but not *DPM2* genes (personal communication with T. Kinoshita). Therefore, D9m271 cells should be *DPM2* mutants.

In this paper, we reported that *C. septicum* alpha toxins were a useful selecting agent to isolate various mutant cells defective in GPI biosynthesis. Previously, mutant cells of *PIG-O*, *PIG-U*, *Gaa1*, *PIG-N*, *PIG-W* and *PIG-V* involved in GPI biosynthesis were easily isolated from CHO cells by aerolysin (Hong *et al.*, 2002; Hong *et al.*, 2003; Murakami *et al.*, 2003; Kang *et al.*, 2005). *C. septicum* alpha toxins binds and destroys the cells with a similar mechanism of aerolysin (Hong *et al.*, 2002; Hong *et al.*, 2003; Murakami *et al.*, 2003). Also, there was a report that *PIG-L* mutant cells were isolated by alpha toxins (Gordon *et al.*, 1999). To isolate different type of GPI-mutant except PIG-L one, we examined whether *C. septicum* alpha toxins can be used as a screening agent and isolated *PIG-W* and *DPM2* mutants cells by alpha toxins. Although we did not find novel mutant, this work indicated that alpha toxins can be used for isolation of various GPI-mutant cells to unravel the function of enzymes responsible for GPI biosynthesis.

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References

Abrami, L., M. Fivaz, and F.G. van der Goot. 2000. Surface dynamics of aerolysin on the plasma membrane of living cells. *Int. J. Med. Microbiol.* 290, 363-367.

Ballard, J., A. Bryant, D. Stevens, and R.K. Tweten. 1992. Purification and characterization of the lethal toxin (alpha-toxin) of *Clostridium septicum*. *Infect. Immun.* 60, 784-790.

Ballard, J., J. Crabtree, B.A. Roe, and R.K. Tweten. 1995. The primary structure of *Clostridium septicum* alpha-toxin exhibits similarity with that of *Aeromonas hydrophila* aerolysin. *Infect. Immun.* 63, 340-344.

Diep, D.B., K.L. Nelson, S.M. Raja, E.N. Pleshak, and J.T. Buckley. 1998. Glycosylphosphatidylinositol anchors of membrane

glycoproteins are binding determinants for the channel-forming toxin aerolysin. *J. Biol. Chem.* 273, 2355-2360.

Eisenhaber, B., S. Maurer-Stroh, M. Novatchkova, G. Schneider, and F. Eisenhaber. 2003. Enzymes and auxiliary factors for GPI lipid anchor biosynthesis and post-translational transfer to proteins. *Bioessays* 25, 367-385.

Ferguson, M.A. 1999. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *J. Cell. Sci.* 112 (Pt 17), 2799-2809.

Fivaz, M., L. Abrami, Y. Tsitrin, and F.G. van der Goot. 2001. Aerolysin from *Aeromonas hydrophila* and related toxins. *Curr. Top. Microbiol. Immunol.* 257, 35-52.

Gordon, V.M., K.L. Nelson, J.T. Buckley, V.L. Stevens, R.K. Tweten, P.C. Elwood, and S.H. Leppla. 1999. *Clostridium septicum* alpha toxins uses glycosylphosphatidylinositol-anchored protein receptors. *J. Biol. Chem.* 274, 27274-27280.

Gowda, D.C. and E.A. Davidson. 1999. Protein glycosylation in the malaria parasite. *Parasitol. Today* 15, 147-152.

Hong, Y., K. Ohishi, J.Y. Kang, S. Tanaka, N. Inoue, J. Nishimura, Y. Maeda, and T. Kinoshita. 2003. Human PIG-U and yeast Cdc91p are the fifth subunit of GPI transamidase that attaches GPI-anchors to proteins. *Mol. Biol. Cell.* 14, 1780-1789.

Hong, Y., K. Ohishi, N. Inoue, J.Y. Kang, H. Shime, Y. Horiguchi, F.G. van der Goot, N. Sugimoto, and T. Kinoshita. 2002. Requirement of N-glycan on GPI-anchored proteins for efficient binding of aerolysin but not *Clostridium septicum* alpha-toxin. *EMBO J.* 21, 5047-5056.

Ikezawa, H. 2002. Glycosylphosphatidylinositol (GPI)-anchored proteins. *Biol. Pharm. Bull.* 25, 409-417.

Kang, J.Y., Y. Hong, H. Ashida, N. Shishioh, Y. Murakami, Y.S. Morita, Y. Maeda, and T. Kinoshita. 2005. PIG-V involved in transferring the second mannose in glycosylphosphatidylinositol. *J. Biol. Chem.* 280, 9489-9497.

Kinoshita, T. and N. Inoue. 2000. Dissecting and manipulating the pathway for glycosylphos-phatidylinositol-anchor biosynthesis. *Curr. Opin. Chem. Biol.* 4, 632-638.

Maeda, Y., S. Tomita, R. Watanabe, K. Ohishi, and T. Kinoshita. 1998. DPM2 regulates biosynthesis of dolichol phosphate-mannose in mammalian cells: correct subcellular localization and stabilization of DPM1, and binding of dolichol phosphate. *EMBO J.* 17, 4920-4929.

Maeda, Y., S. Tanaka, J. Hino, K. Kangawa, and T. Kinoshita. 2000. Human dolichol-phosphate-mannose synthase consists of three subunits, DPM1, DPM2 and DPM3. *EMBO J.* 19, 2475-2482.

Murakami, Y., U. Siripanyapinyo, Y. Hong, J.Y. Kang, S. Ishihara, H. Nakakuma, Y. Maeda, and T. Kinoshita. 2003. PIG-W is critical for inositol acylation but not for flipping of glycosylphosphatidylinositol-anchor. *Mol. Biol. Cell.* 14, 4285-4295.

Nagamune, K., T. Nozaki, Y. Maeda, K. Ohishi, T. Fukuma, T. Hara, R.T. Schwarz, C. Sutterlin, R. Brun, H. Riezman, and T. Kinoshita. 2000. Critical roles of glycosylphosphatidylinositol for *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA* 97, 10336-10341.

Nakamura, N., N. Inoue, R. Watanabe, M. Takahashi, J. Takeda, V.L. Stevens, and T. Kinoshita. 1997. Expression cloning of *PIG-L*, a candidate N-acetylglucosaminyl-phosphatidylinositol deacetylase. *J. Biol. Chem.* 272, 15834-15840.

Parker, M.W., J.T. Buckley, J.P. Postma, A.D. Tucker, K. Leonard, F. Pattus, and D. Tsernoglou. 1994. Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* 367, 292-295.

Quinn, C.P., Y. Singh, K.R. Klimpel, and S.H. Leppla. 1991. Functional mapping of anthrax toxin lethal factor by in-frame insertion mutagenesis. *J. Biol. Chem.* 266, 20124-20130.

Sellman, B.R. and R.K. Tweten. 1997. The propeptide of *Clostridium septicum* alpha toxins functions as an intramolecular chaperone and is a potent inhibitor of alpha toxins-dependent cytolysis. *Mol. Microbiol.* 25, 429-440.

Shin, D.-J., J.J. Lee, H.E. Choy, and Y. Hong. 2004. Generation and characterization of *Clostridium septicum* alpha toxins mutants and their use in diagnosing paroxysmal nocturnal hemoglobinuria. *Biochem. Biophys. Res. Commun.* 324, 753-760.

Sousa, M.V., M. Richardson, W. Fontes, and L. Morhy. 1994. Homology between the seed cytolysin enterolobin and bacterial aerolysins. *J. Protein Chem.* 13, 659-667.

Takeda, J., T. Miyata, K. Kawagoe, Y. Iida, Y. Endo, T. Fujita, M. Takahashi, T. Kitani, and T. Kinoshita. 1993. Deficiency of the GPI-anchor caused by a somatic mutation of the *PIG-A* gene in paroxysmal nocturnal hemoglobinuria. *Cell* 73, 703-711.

Tarutani, M., S. Itami, M. Okabe, M. Ikawa, T. Tezuka, K. Yoshikawa, T. Kinoshita, and J. Takeda. 1997. Tissue-specific knockout of the mouse *Pig-a* gene reveals important roles for GPI-anchored proteins in skin development. *Proc. Natl. Acad. Sci. USA* 94, 7400-7405.

van der Goot, F.G., K.R. Hardie, M.W. Parker, and J.T. Buckley. 1994. The C-terminal peptide produced upon proteolytic activation of the cytolytic toxin aerolysin is not involved in channel formation. *J. Biol. Chem.* 269, 30496-30501.

Ware, F.E. and M.A. Lehrman. 1998. Expression cloning of a novel suppressor of the Lec15 and Lec35 glycosylation mutations of Chinese hamster ovary cells. *J. Biol. Chem.* 273, 13366.