The glycan core of GPI-anchored proteins modulates aerolysin binding but is not sufficient: the polypeptide moiety is required for the toxin–receptor interaction

Laurence Abrami[a], Marie-Claire Velluz[a], Yeongjin Hong[b], Kazuhito Ohishi[b], Angela Mehlert[c], Michael Ferguson[c], Taroh Kinoshita[b], F. Gisou van der Goot[a,*]

[a] *Department of Genetics and Microbiology, University of Geneva, 1 rue Michel Servet, 1211 Geneva 4, Switzerland*
[b] *Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan*
[c] *Division of Biological Chemistry and Molecular Microbiology, School of Life Sciences, University of Dundee, Dundee, UK*

Received 17 December 2001; revised 7 January 2002; accepted 8 January 2002

First published online 18 January 2002

Edited by Guido Tettamanti

Abstract Sensitivity of mammalian cells to the bacterial toxin aerolysin is due to the presence at their surface of glycosylphosphatidyl inositol (GPI)-anchored proteins which act as receptors. Using a panel of mutants that are affected in the GPI biosynthetic pathway and *Trypanosoma brucei* variant surface glycoproteins, we show that addition of an ethanolamine phosphate residue on the first mannose of the glycan core does not affect binding. In contrast, the addition of a side chain of up to four galactose residues at position 3 of this same mannose leads to an increase in binding. However, protein free GPIs, which accumulate in mutant cells deficient in the transamidase that transfers the protein to the pre-formed GPI-anchor, were unable to bind the toxin indicating a requirement for the polypeptide moiety, the nature and size of which seem of little importance although two exceptions have been identified.

Key words: Aerolysin; Glycosylphosphatidyl inositol; Variant surface glycoprotein; Pore forming; Lectin

1. Introduction

Aerolysin is one of the major virulence factors produced by *Aeromonas hydrophila* [1]. It has no enzymatic activity but has the capacity to form pores in the plasma membrane of targets cells [2]. All mammalian cells so far tested are sensitive to aerolysin due to the ubiquitous expression of its receptors. Interestingly, aerolysin does not appear to bind to a specific polypeptide chain since it recognizes a post-translational modification, a glycosylphosphatidyl inositol (GPI) anchor [3–5]. This anchor is added, in the endoplasmic reticulum, to the carboxy-terminus of newly synthesized proteins that bear a GPI-anchoring signal [6,7]. The GPI anchor then targets these proteins to the plasma membrane. All anchors, from yeast to mammals, have the same backbone structure consisting of ethanolamine-HPO_4-6Manα1-2Manα1-6Manα1-4Glc$NH_2\alpha$1-6-myo-inositol-1HPO_4 linked to a lipid moiety (Fig. 1A). Whereas in some parasites such as *Leishmania* this represents the complete anchor, the core is modified by one or more side chains in trypanosomes and mammalian cells [8]. The GPI anchor appears to be necessary for aerolysin binding since addition of a mammalian anchor to cathepsin D, which is unable to bind the toxin, was shown to transform this protein into an aerolysin binding protein [5]. The naked anchor (having no side chain modifications), consisting only of the conserved core, however, appeared to be insufficient for binding. Diep et al. [5] indeed failed to detect binding of aerolysin on blots of the *Leishmania* GPI-anchored protein gp63 when expressed in the parasite, whereas binding on blots was observed when gp63 was expressed in Chinese hamster ovary (CHO) cells. Our interest here was to study the sugar modifications within the GPI anchor that are required for aerolysin binding.

2. Materials and methods

2.1. Cells and materials

Baby hamster kidney (BHK), HeLa, CHO, F9 and K562 cells were grown as previously described [4,9–12]. Pig-a (F9 Pig-a), Gaa1 (F9 Gaa1) and Pig-n (F9 Pig-n) knockout F9 cells were generated by means of homologous recombination [11,13]. They are respectively deficient in the enzyme that transfers GlcNAc from UDP-GlcNAc to phosphatidyl inositol (Pig-a), in a component of the GPI transamidase complex (Gaa1), and in the enzyme responsible for the addition of a phosphoethanolamine to the first mannose of the GPI precursor (Pig-n). Class A and class K GPI-anchor-deficient K562 cells [12,14] were gifts from S. Hirose and M.E. Medof (Case Western Reserve University, Cleveland, OH, USA). CHO IIIB2A were obtained after stable transfection of CHO cells with the human CD55 and CD59 cDNAs [15].

Proaerolysin was purified [4,16] and labeled with Alexa 546 according to the manufacturer's instructions (Molecular Probes) or with ^{125}I (Amersham Pharmacia Biotech) as previously described [4]. Proaerolysin was treated with trypsin (1:20, enzyme protein ratio) for 10 min at room temperature leading to aerolysin. Soluble forms of two variable surface glycoprotein (VSG) variants, sVSG117 and sVSG118, were purified from trypanosomes as described [17] with minor modifications [18]. In contrast to the 118 variant [19], the sVSG117 variant has a side chain of up to four galactose residues at position 3 of the

*Corresponding author. Tel.: (41)-22-702 5652; Fax: (41)-22-702 5702.
E-mail address: gisou.vandergoot@medecine.unige.ch (F. Gisou van der Goot).

Abbreviations: CHO, Chinese hamster ovary; GPI, glycosylphosphatidyl inositol; IM, incubation medium; PBFI, potassium binding benzofuran isophthalate; VSG, variant surface glycoprotein

0014-5793/02/$22.00
PII: S0014-5793(02)02274-3

first mannose (Fig. 1A) [20]. Anti-CD52, anti-Thy-1 and anti-CD59 antibodies were gifts from H. Waldmann (Oxford) [21], C. Bron (Lausanne) [22] and M. Tomita [23], respectively. Purified gp63 from *Leishmania major* L119 was a gift from Dr. K. Herrmann (IBFH, Leipzig, Germany).

2.2. Proaerolysin binding

Confluent cells were washed three times with ice-cold PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^{2+}). Cells were then incubated at 4°C with 0.4 nM aerolysin or 0.96 nM [^{125}I]aerolysin in incubation medium (IM) containing Glasgow minimal essential medium buffered with 10 mM HEPES, pH 7.4, for 1 h. Cells were washed three times for 5 min with PBS^{2+} at 4°C. Cells were subsequently washed with ice-cold PBS^{2+}, scraped from the dish, and collected by centrifugation. The presence of cell-bound aerolysin was analyzed by Western blotting and that of cell-bound [^{125}I]proaerolysin by counting.

2.3. Transfection and immunofluorescence

In order to analyze the ability of aerolysin to bind to CD52, the human CD52 DNA was cloned in the eukaryotic expression vector pcDNA3, purified using Quiagen columns and transfected into HeLa cells using the Fugene transfection reagent (Roche). The presence of aerolysin binding components in various cell types (transfected or not) was analyzed by fluorescence microscopy in the following manner. Cells grown on cover slips were fixed with 3% paraformaldehyde, permeabilized or not with 0.1% saponin and labeled with 7.5 nM Alexa–aerolysin. Fluorescence microscopy was carried out using an inverted Axiovert 135 TV Zeiss microscope equipped with a cooled-CCD camera (Princeton Instruments), driven by the IP lab imaging system software.

2.4. Potassium efflux measurements

Potassium efflux measurements were performed as described [4]. Briefly, confluent cell monolayers were incubated with aerolysin for various times at 37°C in IM, then washed with ice-cold potassium-free choline medium, pH 7.4, containing 129 mM choline–Cl, 0.8 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM citric acid, 5.6 mM glucose, 10 mM NH_4Cl, 5 mM H_3PO_4 and solubilized with 0.5% Triton X-100 in the same buffer for 20 min at 4°C. The potassium content of the cell lysates was determined by flame photometry using a Philips PYE UNICAM SP9 atomic absorption spectrophotometer. The intracellular potassium is calculated as a fraction of potassium content of untreated cells.

2.5. Analytical techniques

SDS–PAGE was performed using the Laemmli buffer system. Aerolysin overlays were performed as previously described [4,10]. Overlays can be indifferently performed with proaerolysin and aerolysin. For reasons of clarity the term aerolysin will be used throughout the text. After performing overlays using [^{125}I]aerolysin, binding was quantified using a Bio-Rad phosphorimager (Molecular Imager FX) driven by the Quantify One (v 4.2.1) software. Protein concentrations of cellular fractions were determined with bicinchoninic acid (Pierce). Labeling of GPI anchors with [^{3}H]mannose (Amersham Pharmacia Biotech) was performed as described by Hirose et al. [14]. Potassium efflux was measured using the fluorescent dye PBFI (potassium binding benzofuran isophthalate) as previously described [24].

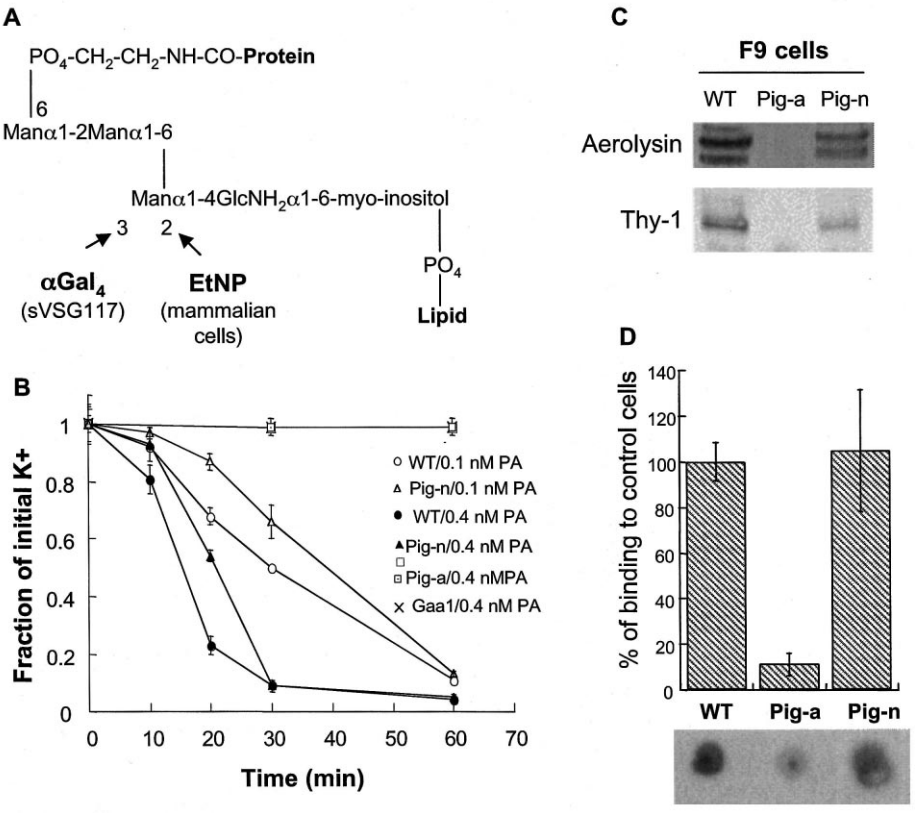

Fig. 1. Effect of the Pig-n mutation on binding and channel formation by aerolysin. A: Chemical structure of the GPI glycan core. Two, amongst the many, glycan core modifications are indicated: addition of an EtNP on the first mannose such as in many mammalian anchors, and addition of a four-galactose residue side chain on the first mannose as in VSG117. B: Aerolysin-induced potassium efflux from wild type, Pig-n but not Pig-a and Gaa1 mutant F9 cells. Cells were incubated for various times with 0.1 or 0.4 nM aerolysin. The intracellular potassium content was then determined by flame photometry ($n = 3$, error bars represent standard deviations). C: Aerolysin binds to wild type and F9 Pig-n cells. Wild type and Pig-n mutant F9 cells were incubated with 0.4 nM aerolysin for 1 h at 4°C. Post-nuclear supernatants were then prepared and analyzed by Western blotting for the presence of aerolysin (the double bands represent the precursor aerolysin and mature furin-cleaved aerolysin). Binding to F9 Pig-a was used as a negative control. The levels of Thy-1 expression of these cells were probed by Western blotting. D: Binding of aerolysin to extracts of wild type and F9 Pig-n cells was analyzed by toxin overlay on dot blots of cell extracts. Overlays were performed using [^{125}I]aerolysin and binding was quantified ($n = 3$, error bars represent standard deviations).

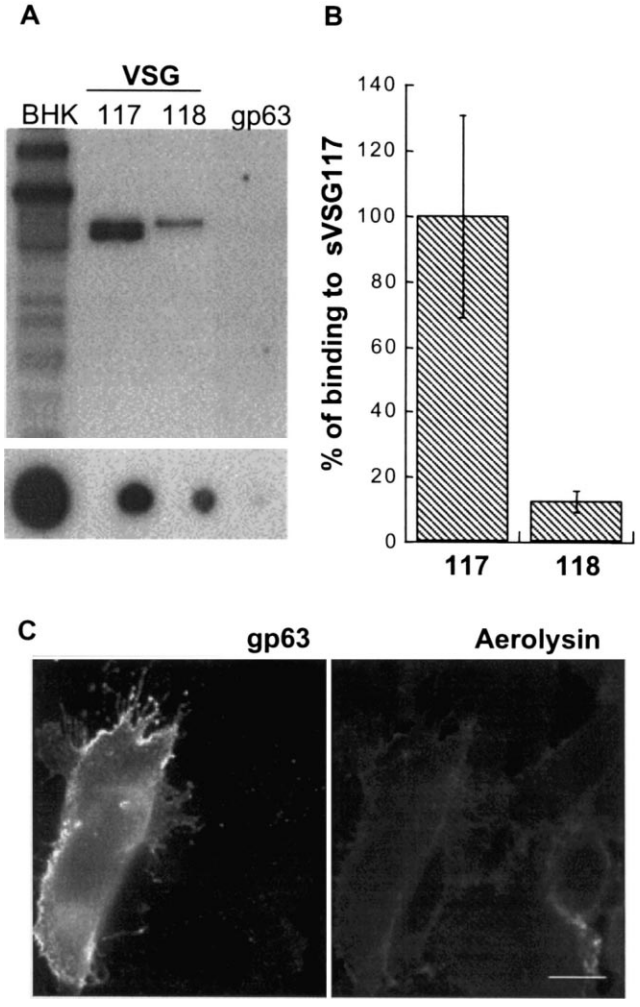

Fig. 2. Aerolysin binds to GPI-anchored proteins with naked anchors. The ability of aerolysin to bind to 200 ng of purified trypanosomal sVSG117, sVSG118 and to gp63 from *Leishmania* was analyzed by toxin overlay either after SDS–PAGE or after dot blotting the proteins (A). B: Binding of [^{125}I]aerolysin to sVSG117 and sVSG118 on dot blots was quantified ($n = 3$, error bars represent standard deviations). C: CHO cells were transfected with the *Leishmania* gp63 gene. Transfected cells were identified by labeling with an anti-gp63 antibody. When paraformaldehyde-fixed cells were co-labeled with 7.5 nM Alexa-aerolysin, transfected cells did not show any increase in aerolysin binding. Bar = 10 μm.

3. Results

3.1. The role of GPI-anchor mannose modifications in aerolysin binding

In order to identify the sugar modifications required for aerolysin binding to the GPI anchor, we first analyzed a mutant cell line, F9 Pig-n, affected in the Pig-n gene. These cells express GPI anchors that have no ethanolamine phosphate (EtNP) group on the first mannose [13] (Fig. 1A), whereas most wild type mammalian anchors do. To test whether mutant cells were still sensitive to the toxin, we measured aerolysin-induced potassium efflux. Both wild type and mutant F9 cells were sensitive to aerolysin, in contrast to GPI-deficient F9 Pig-a cells, that are affected in the first step of GPI biosynthesis [6] (Fig. 1B). The kinetics of potassium efflux were however slower for F9-Pig-n cells, in agreement with the previous observation that these cells express lower amounts of

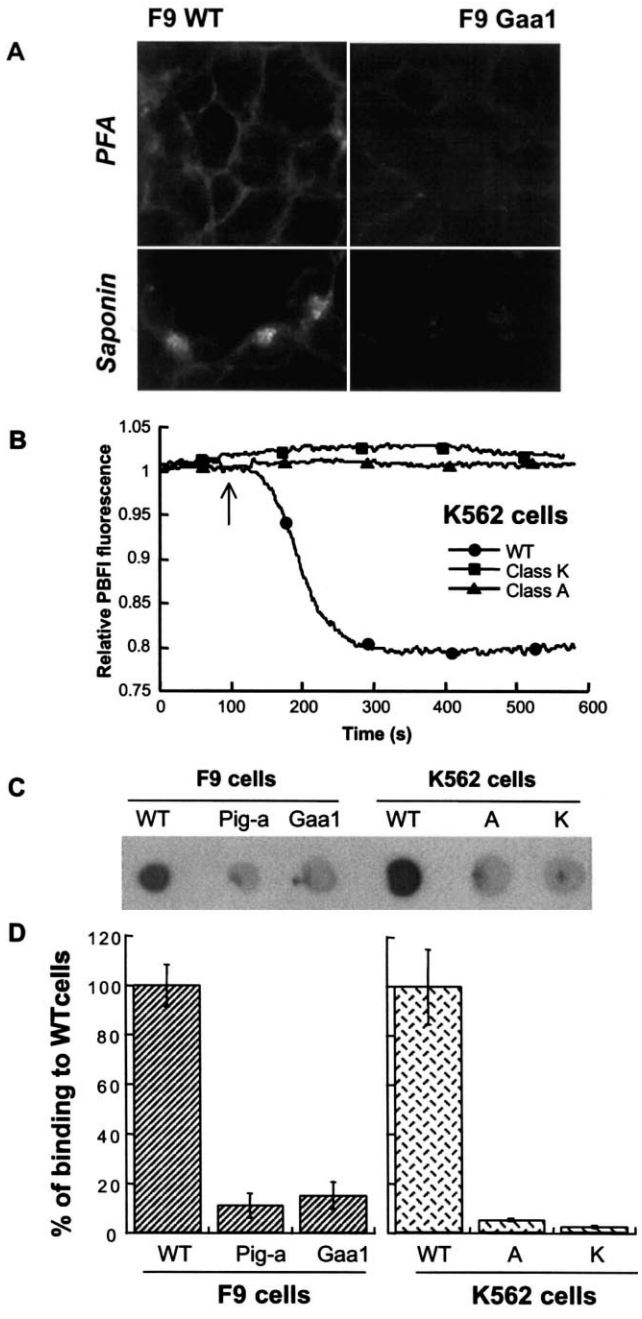

Fig. 3. Aerolysin does not bind to free GPIs. A: Binding of Alexa-labeled aerolysin to wild type and Gaa1 mutant F9 cells was analyzed by fluorescence microscopy. Cells were fixed with 3% paraformaldehyde, permeabilized or not with 0.1% saponin and labeled with 7.5 nM Alexa-aerolysin. B: Sensitivity to aerolysin was analyzed by measuring the permeabilization of the plasma membrane to potassium using the K^+-sensitive dye PBFI. Aerolysin was added at the time indicated by the arrow (100 ng/ml). C: Binding of aerolysin to cell extracts of wild type, Pig-a and Gaa1 mutant F9 cells as well as to wild type class A and class K K562 cells was analyzed by toxin overlay on dot blots. In order to load the same amounts of GPI anchors, irrespective of the presence or absence of attached proteins, cells were labeled with [^{3}H]mannose in the presence of tunicamycin and equal counts were dot blotted. D: Binding of [^{125}I]aerolysin was quantified ($n = 3$, error bars represent standard deviations).

Thy-1 [13] and probably other GPI-anchored proteins. That aerolysin did bind to F9 Pig-n cells was confirmed by fluorescence analysis of cells that had been incubated with fluorescent aerolysin (not shown), by Western blot analysis of total cell extracts (Fig. 1C) and by aerolysin overlay of dot blots of cell extracts (Fig. 1D). In this latter experiment, cells were labeled with [^{3}H]mannose in the presence of tunicamycin in order to specifically label GPI anchors. The same number of counts were dot blotted, presumably corresponding to the same total amount of GPI anchors. Quantification indicates that binding to wild type and Pig-n F9 is very similar, indicating that the ethanolamine residue at position 2 of the first mannose is not essential for binding of aerolysin to the glycan core and does not affect the affinity of the toxin for the anchor.

In order to confirm that modification of the first mannose residue is not mandatory for aerolysin binding, we made use of the observation that aerolysin binds, in vitro, to the VSG of bloodstream forms of *Trypanosoma brucei* [5] and of the availability of well-characterized variants of this protein [25]. The sVSG117 variant was shown to have a single modification at the conserved core, i.e. a side chain of up to four galactose residues at position 3 of the first mannose (Fig. 1A) [20], which is absent from the 118 variant [19]. As shown in Fig. 2, aerolysin binds to both variants. Binding is however far more pronounced with the 117 variant, suggesting that the modification at position 3 of the first mannose increased the binding affinity.

Interestingly, binding to sVSG118 also reveals that aerolysin is able to bind to a naked anchor EtNP–Man_3GlcN-PI with no side chain modifications. Aerolysin was however unable to bind on blots of *Leishmania* gp63, a GPI-anchored protein that also has a naked anchor [26] (Fig. 2), in agreement with previous observation [5]. Similarly, we were unable to detect an increase in binding of aerolysin to CHO cells overexpressing *Leishmania* gp63 (Fig. 2C), indicating that even with a mammalian anchor (i.e. with side chain modifications), surface-expressed gp63 was not binding competent.

3.2. Aerolysin does not bind to free GPIs

Since the GPI anchor appears to be necessary for aerolysin binding [5], we next investigated whether it was sufficient for binding, i.e. can aerolysin bind to free GPIs. These glycolipid intermediates accumulate in cells, such as F9 Gaa1 mutants, that are deficient in the transamidase complex involved in adding the protein moiety onto the pre-formed GPI anchor [11]. Free GPIs are found intracellularly but also reach the extracellular leaflet of the plasma membrane [27]. Despite the documented presence of free GPIs at the cell surface, we found that F9 Gaa1 mutants were not sensitive to aerolysin (Fig. 1B). Also, we were unable to detect any labeling on fixed and permeabilized F9 Gaa1 mutant cells using an aerolysin fluorescent derivative, in contrast to what is observed on wild type cells (Fig. 3A), indicating that aerolysin is also incapable of binding to intracellular free GPIs. Finally, we also failed to detect significant binding by toxin overlay on dot blots of cell extracts (Fig. 3C,D). In these experiments, cells were labeled with radioactive mannose in the presence of tunicamycin in order to label GPI anchors, and equal counts were dot blotted. These experiments indicate that aerolysin is unable to bind to free GPIs. Similarly, human leukemic K562 cells deficient in the GPI transamidase were resistant to aerolysin (Fig. 3B) and the toxin failed to bind to extracts of these cells after dot blot (Fig. 3C,D).

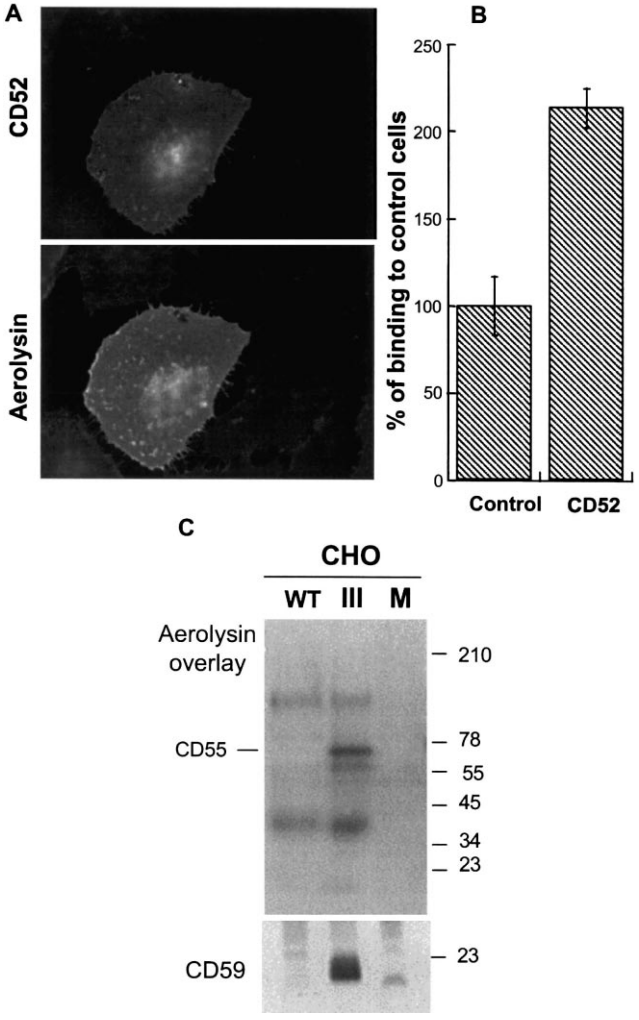

Fig. 4. Aerolysin binds to CD52 but not to CD59. A,B: HeLa cells were transfected with the human CD52 gene. Transfected cells were identified by labeling with an anti-CD52 antibody (A). When paraformaldehyde-fixed cells were co-labeled with 7.5 nM Alexa-aerolysin, transfected cells showed dramatically increased aerolysin binding (A). B: The effect on CD52 transfection on binding of [^{125}I]aerolysin to living cells was quantified ($n = 6$, error bars represent standard deviations). Note that the efficiency of transfection into these HeLa cells was approx. 60%. C: Extracts of CHO cells stably transfected (CHO IIIB2A, labeled III) [15] or not (WT) with the human CD55 and CD59 genes were analyzed by aerolysin overlay and Western blotting using an anti-CD59 antibody. An extract from cells deficient in GPI biosynthesis (CHO M2S2, labeled M) were analyzed as a control.

3.3. The protein moiety of the GPI-anchored polypeptide is required for aerolysin binding

The above-described experiments show that the GPI anchor is not sufficient for aerolysin binding and that the protein needs to be present. We next tested whether the toxin was able to bind to the smallest known GPI-anchored protein CD52 that is composed of only 12 amino acids [28]. HeLa cells were transfected with the human CD52 gene, fixed, and incubated with Alexa labeled aerolysin. As shown in Fig. 4A, transfected cells were characterized by a dramatically increased aerolysin binding. A more than two-fold increase in

binding of [^{125}I]aerolysin to living CD52 transfected cells was observed (transfection rate $\approx$ 60%; Fig. 4B). This observation however does not imply that all GPI-anchored proteins of 12 amino acids or more will bind aerolysin. Indeed, during the course of our studies, we encountered two GPI-anchored proteins that were unable to act as an aerolysin receptor: gp63, as mentioned above, and CD59. We were unable to detect CD59 by aerolysin overlays on extracts of CHO cells stably transfected with the human CD59 gene [15], whereas CD55, the gene of which had been co-transfected was readily labeled (Fig. 4C).

4. Discussion

Previous observations have shown that the presence of a GPI anchor is required for aerolysin binding [3–5]. Binding will occur in the absence of the lipid moiety of the anchor as illustrated here by the binding of aerolysin to sVSGs, which had their diacylglycerol moieties removed by PI-PLC (Fig. 2) in agreement with previous observations [4,5]. Here we show that naked anchors are binding competent, but that certain modifications of the first mannose of the conserved core can modulate binding. Addition of an EtNP group at position 2 had no effect. In contrast, binding was promoted by the presence of an up to four-galactose side chain at position 3.

Despite its importance, the GPI anchor alone is not sufficient for aerolysin binding as indicate by the absence of binding to free GPIs. This finding came somewhat as a surprise, since aerolysin was shown to bind to a variety of GPI-anchored proteins that have no sequence homology, such as thy-1 [3], contactin [5], and CD14 (Fivaz et al., submitted). More strikingly, we have shown that in BHK cells aerolysin binds to all GPI-anchored proteins (approx. 10) (Fivaz et al., submitted). These observations strongly suggest that the toxin does not discriminate between the proteins present on the GPI-anchors. We however believe that GPI-anchored proteins that do not allow aerolysin binding remain the exception since we have only encountered two such proteins during the course of our studies, CD59 and gp63.

The lack of sequence homology between identified aerolysin binding proteins suggests that there is not direct contact between the toxin and the polypeptide chain. To explain the role of the protein moiety in aerolysin binding, at least four, not mutually exclusive, possibilities can be proposed. (1) Aerolysin could recognize some post-translational modification. This could be *N*-linked glycosylation since most if not all known GPI-anchored proteins are glycosylated and often heavily glycosylated. The position of the *N*-linked sugars would however have some importance since both CD59 and gp63 are glycosylated [29,30] but neither one permits toxin binding. (2) The conformation of the GPI anchor that allows aerolysin binding is acquired only when a polypeptide is attached. This is not unlikely since the lipid moiety has been shown to influence the antigenicity of some GPI-anchored proteins [28,31,32]. Therefore, lipid, glycan core and polypeptide appear to influence each other's structure. The conformation of the glycan core could then differ depending on the structure of this polypeptide and some rare conformations would not allow aerolysin binding. (3) The amide bond between the GPI anchor and the protein C-terminus or (4) the higher order assembly (multimerization for example) of the GPI-anchored proteins could be important for aerolysin binding. This later possibility is suggested by the fact that aerolysin was found to bind to gp63 expressed in CHO cells on blots [5] but not on living cells (Fig. 2C). These issues await further investigations and hopefully the analysis of aerolysin-resistant mutant cells will allow the unraveling of aerolysin binding determinants.

Acknowledgements: We would like to thank S. Hirose and M.E. Medof for sharing their cell lines, and H. Waldmann, C. Bron and M. Tomita for providing us with cDNAs and antibodies. This work was supported by a grant from the Swiss National Science Foundation to G.v.d.G.

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