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# Adventures of a pore-forming toxin at the target cell surface

**Laurence Abrami, Marc Fivaz and F. Gisou van der Goot**

**The past three years have shed light on how the pore-forming toxin aerolysin binds to its target cell and then hijacks cellular devices to promote its own polymerization and pore formation. This selective permeabilization of the plasma membrane has unexpected intracellular consequences that might explain the importance of aerolysin in *Aeromonas* pathogenicity.**

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Aerolysin is one of the major toxins secreted by the Gram-negative bacterium *Aeromonas hydrophila*, as well as other *Aeromonas* species that are human pathogens involved in food-borne infections[1]. In a mouse toxicity model, mutant strains deficient in aerolysin production were found to be less virulent than wild type[2]. Additionally, specific neutralizing antibodies to aerolysin have been detected in animals surviving *Aeromonas* infection.

Aerolysin is released from *A. hydrophila*, via a type II secretion system, as a soluble precursor, proaerolysin[1], which undergoes a monomer-to-dimer transition at high concentrations such as those found during crystallization processes[3,4]. The structure of proaerolysin indicates that it consists of two lobes[3] (Fig. 1): the amino-terminal domain 1 (Ref. 5) and a less stable, large carboxy-terminal region that can be divided into three domains, domains 2–4 (Fig. 1). Domain 1 shares structural homology with the S2 and S3 subunits of the *Bordetella pertussis* toxin and is similar to a fold found in C-type lectins[6] (blue in Fig. 1). In addition, proaerolysin shares strong sequence homology with *Clostridium septicum* alpha toxin, which has a similar mode of action[7]. Interestingly, the proaerolysin sequence contains no hydrophobic stretches predicted to be capable of membrane insertion.

## Receptor binding and activation

Once released by the bacterium, proaerolysin binds to high-affinity receptors on the target cell. Instead of recognizing one specific receptor protein, proaerolysin interacts with a specific post-translational modification, a glycosylphosphatidylinositol (GPI) anchor[8–12]. This anchor is added, in the endoplasmic reticulum (ER), to the carboxy terminus of newly synthesized proteins bearing a GPI-anchoring signal. The GPI anchor then targets these proteins to the plasma membrane[13]. All anchors have the same backbone structure consisting of ethanolamine-$HPO_4$-6Manα1-2Manα1-6Manα1-4Glc$NH_2$α1-6-myo-inositol-1$HPO_4$ linked to a lipid moiety. The mannose residues can be modified and the inositol ring can be acylated in certain cell types. Binding of proaerolysin appears to involve mainly the glycan core and probably specific modifications of the central mannose residues[10]. To what extent the protein moiety of the receptor influences binding remains to be established. However, it

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is clear that proaerolysin binds to most, if not all, GPI-anchored proteins expressed on the surface of a given cell type.

The affinity of proaerolysin for its receptors has been shown to be approximately 20 nM by *in vivo* studies[11] as well as by surface plasmon resonance[14]. Proaerolysin from which domain 1 has been removed interacts with GPI-anchored proteins with an affinity that is 50 times weaker than that of the full-length toxin, indicating that domain 1 is crucial for high-affinity binding but that domains 2–4 also make a contribution[14,15]. This was confirmed by the identification of specific mutations in domains 1 and 2 that affect interaction[14] (red in Fig. 1). Whether all identified amino acids directly interact with the GPI anchor remains to be seen. It is striking, however, that many of the residues identified as important for binding are aromatic residues. Similar observations have been made for other carbohydrate-binding proteins in which aromatic residues have been shown to stack against the pyranose rings of sugars[16]. It is tempting to speculate that both domains 1 and 2 of aerolysin bind the anchor and that it is the presence of two binding sites that leads to the high overall affinity of proaerolysin for its receptor.

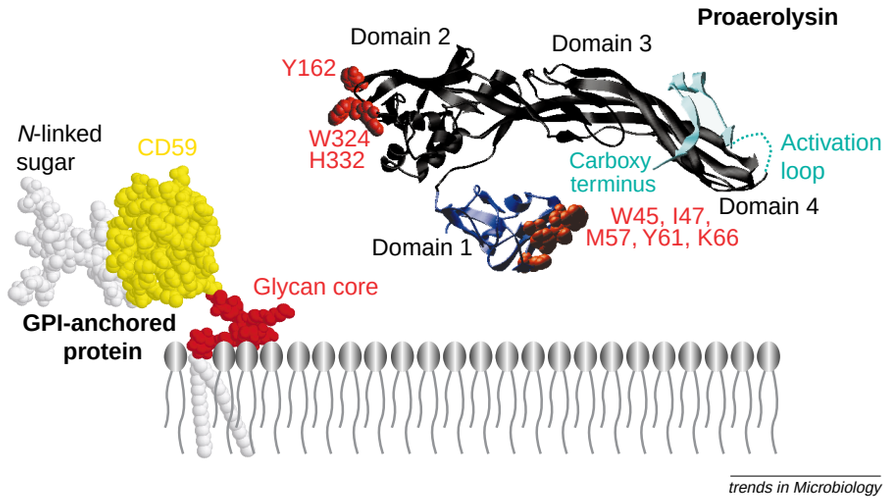


**Fig. 1.** Proaerolysin and its glycophosphatidylinositol (GPI) glycan core receptor. A ribbon diagram of proaerolysin, based on its X-ray structure[3], a molecular model of GPI-anchored CD59, based on the protein coordinates of the solution structure and the characterization of the GPI anchor and an *N*-linked sugar[17] are shown. In the proaerolysin molecule, the activation loop and the activation peptide are shown in light blue. The amino acids that have been identified as being involved in receptor binding are shown in red[14]. For CD59, the glycan core, also in red, is shown as modelled by Rudd *et al.*[17] However, the acyl chain on the inositol ring, found in human erythrocytes, but rarely on other cell types, has been removed. Only one *N*-linked sugar is shown. The protein moiety of CD59 is shown in yellow.

How proaerolysin binds to the glycan core of a GPI-anchored protein is unclear. One well characterized glycan core is that of CD59, a model of which is shown in Fig. 1 (Ref. 17). As can be seen, the glycan core is small with respect to proaerolysin and little space is available for the toxin to slip in-between the membrane surface and the proteinaceous moiety. Recent evidence suggests that the proaerolysin monomer, and not the dimer as previously thought, is the receptor-binding form[4]. Unfortunately, only the 3-D structure of the dimer is available. The structure of the monomer could well differ from that of the dimer and thus the position of domain 1 with respect to the large lobe could be different from that depicted in Fig. 1. Nevertheless, it remains difficult to envision how domains 1 and 2 can interact with the glycan core of the GPI anchor. Conformational changes in the glycan core of the anchor and in the toxin are likely to be necessary.

Although the glycan core of a GPI anchor, being very close to the membrane, might allow ideal positioning of aerolysin when it comes to forming a transmembrane channel, it is clearly not the most exposed cell surface motif for a toxin to bind to. Buckley and co-workers have put forward the interesting possibility that, in order to cross the thick layer of glycocalyx covering epithelial cells, aerolysin initially binds to surface oligosaccharides with low affinity, possibly through its lectin-like domain 1 or other tryptophan-rich regions of the molecule, before being progressively shuttled to the membrane vicinity where it would bind the glycan core of the GPI anchor[14]. However, this attractive relay mechanism, involving low- and high-affinity binding sites, remains to be proven.

Once receptor bound, proaerolysin is processed into aerolysin either by soluble digestive enzymes or by the transmembrane mammalian endoprotease furin[18] (Fig. 2). Even though furin is found predominantly in the trans-Golgi network, it cycles with the cell surface, and it has been demonstrated that processing occurs at the plasma membrane and does not require toxin internalization[18]. As GPI-anchored proteins diffuse though the glycerophospholipid region of the plasma membrane at the same rate as lipids rather than at the same rate as transmembrane proteins, this type of receptor is likely to increase the encounter rate between proaerolysin and furin[19].

## Oligomerization

Mature aerolysin must then undergo circular oligomerization into a heptameric complex, which is the channel-forming configuration. The formation of this complex is extremely efficient at the surface of living cells in contrast to what is observed in solution. As aerolysin binds to its receptors with high affinity[11,14], binding will concentrate the toxin at the cell surface, owing to the shift from a 3-D environment to a 2-D environment. However, we have calculated that this change is insufficient to account for the observed difference in oligomerization efficiency[19]. Recently, we have found that aerolysin 'hijacks' a

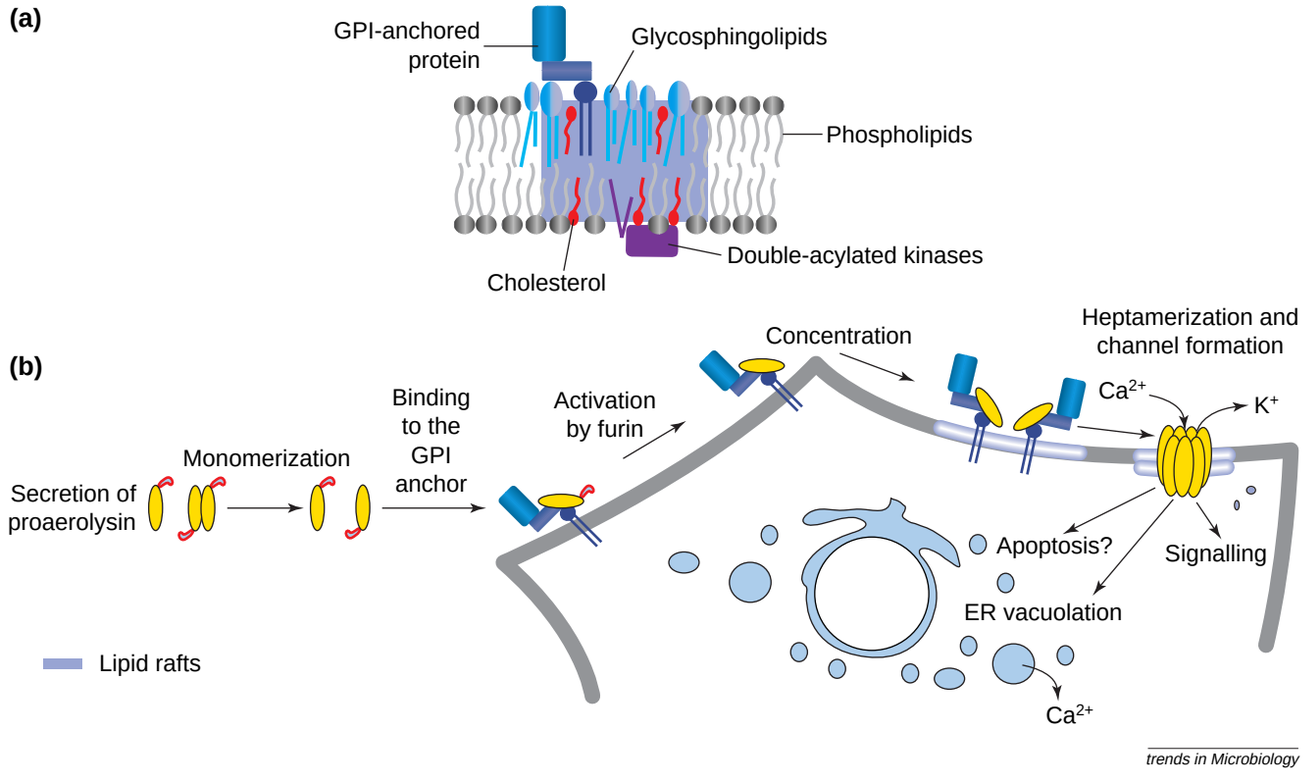


**Fig 2. (a)** Schematic representation of a lipid raft. These domains have been shown to be enriched in glycosphingolipids, cholesterol, sphingomyelin and lipid-anchored proteins[20]. Rafts have also been shown to be rich in signalling molecules, and in particular to contain doubly acylated tyrosine kinases of the SRC family. **(b)** Channel formation by aerolysin and its intracellular effects. The proaerolysin monomer binds to the glycan core of glycophosphatidylinositol (GPI)-anchored proteins on mammalian cells. Proaerolysin is then processed to aerolysin by furin or soluble digestive enzymes. The mature toxin is able to polymerize into a heptameric ring. The transient association of receptor-bound aerolysin with lipid rafts favours this step. This cellular concentration device promotes channel formation. The aerolysin channel selectively permeabilizes the plasma membrane to small ions. As a consequence, several events can occur intracellularly: (1) calcium can be released from the endoplasmic reticulum (ER) via G-protein activation and the PtdIns(3,4,5)*P*3 pathway; (2) selective vacuolation of the ER can be observed, and (3) apoptosis is triggered in certain cell types such as T cells.

concentration device present at the surface of mammalian cells[19]. Binding to GPI-anchored molecules, rather than to transmembrane receptors, not only allows proaerolysin to diffuse more rapidly through the glycerophospholipid region of the plasma membrane where furin is located[19], but it also allows aerolysin to associate transiently with cholesterol-rich microdomains of the plasma membrane, so-called lipid rafts (Fig. 2) (reviewed in Ref. 20). This association leads to local increases in toxin concentration, which in turn allow oligomerization of the toxin under conditions where the average monomer concentration at the plasma membrane is below the threshold required for complex formation. It is not clear at present whether, in addition to providing a concentration platform, the lipid and protein composition of rafts favours oligomerization.

The aerolysin heptamer is then thought to insert spontaneously into the lipid bilayer owing to the exposure of hydrophobic regions[21]. This step is very poorly understood. It has been suggested that unfavourable energetic effects exist at the junctions between lipid rafts and the fluid phase phosphoglyceride region of the plasma membrane[22]. These unstable boundaries, or other particularities of lipid rafts, might facilitate membrane insertion of the aerolysin heptamer.

At present, the structure of the channel is unknown. However, considering the similarities between the modes of action of staphylococcal α-toxin and aerolysin, and the high β-sheet content of these two proteins, it is likely that the transmembrane region of the aerolysin channel is a β-barrel[23], as originally suggested by Parker *et al.*[3]

## Channel formation and intracellular effects

Channel formation by aerolysin leads to the selective permeabilization of the plasma membrane to small ions, such as potassium and calcium, but not proteins. At present, we have been unable to observe any repair of plasma membrane lesions in contrast to what has been described for other toxins. This could be a result of the extraordinary stability of the aerolysin heptamer, its resistance to proteolysis[5] and degradation. Indeed, owing to interactions between domains 3 and 4, the aerolysin heptamer is totally resistant to boiling and chaotropic agents[5]. Alternatively, aerolysin might not be internalized by cells and therefore not be removed from the surface.

In the presence of aerolysin, cells remain viable for several hours (as measured by propidium iodine

exclusion[11]), depending on the toxin concentration. However, channel formation induces a number of cellular responses. In human granulocytes, aerolysin was shown to trigger release of calcium from the ER by activation of G proteins and production of inositol(1,4,5)-trisphosphate[24]. The mechanism by which channel formation by aerolysin induces G-protein activation is unclear. One possibility is that it affects the integrity of the lipid rafts, which have been implicated in modulating and integrating signalling events at the plasma membrane. In T cells, channel formation by aerolysin has been shown to trigger apoptosis, presumably as a result of the massive influx of calcium, a process that can be overcome by overexpression of the anti-apoptotic protein BCL-2 (Ref. 25). Finally, in a variety of polarized and non-polarized epithelial cells, aerolysin has been shown to trigger vacuolation of the ER, a process that cannot be rescued by BCL-2 overexpression[11]. Vacuolation is restricted to the first compartment of the biosynthetic pathway, as neither the morphology of the Golgi complex nor that of endocytic compartments is altered by aerolysin. Vacuolation leads to an arrest in the transport of newly synthesized proteins out of the ER (Ref. 11). It is, however, not known whether this is owing to an effect on protein folding and/or quality control, vesicular transport out of the ER, or both. Vacuolation is inhibited by ATP depletion of cells or depolymerization of the microtubule network, indicating that the process is dependent on the dynamic properties of ER membranes, perhaps because aerolysin interferes with normally occurring ER fission events[11]. However, it is not clear how this inhibition is achieved, especially as there is no evidence that the toxin enters the target cell.

As already discussed, aerolysin induces different downstream effects depending on the cell type. These various events might, however, be triggered initially by a common mechanism: pore formation followed by membrane depolarization, calcium efflux or both. However, it is important to note, that streptolysin O (which also leads to membrane depolarization and calcium influx), calcium ionophores and potassium ionophores do not lead to ER vacuolation as occurs with aerolysin[11].

## Conclusion

This description of the adventures of aerolysin at the target cell surface illustrates how a toxin can make use of specialized membrane lipids as cell surface receptors, in this case the common glycolipid anchor of any GPI-linked protein, to find its processing enzyme, then associate with lipid rafts to promote toxicity. A similar strategy could well be used by other toxins, including pore formers, as well as bacteria and viruses[26]. Cellular components (proteins and lipids) required for channel formation by a number of toxins have indeed been identified as raft components. These include cholesterol, which is required for channel formation by cholesterol-dependent toxins (formerly called thiol-activated toxins) such as perfringolysin O (Ref. 27) and sphingomyelin, which is required for channel formation by both *Eidenia foetida* lysenin[28] and *Vibrio cholerae* cytolysin[29]. Receptors for certain bacteria have also been identified as raft components[30,31].

### Questions for future research

- How does proaerolysin interact with the glycan core in the vicinity of the lipid bilayer?
- Aerolysin oligomerization presumably induces clustering of its GPI-anchored receptors. What are the consequences of this clustering for the target cell?
- What is the mechanism that leads to selective vacuolation of the endoplasmic reticulum?
- How does aerolysin induce apoptosis?
- Why is aerolysin required for the spreading and maintenance of *Aeromonas hydrophila* infections?

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# PII signal transduction proteins


## Alexander J. Ninfa and Mariette R. Atkinson



**PII proteins, found in Bacteria, Archaea and plants, help coordinate carbon and nitrogen assimilation by regulating the activity of signal transduction enzymes in response to diverse signals. Recent studies of bacterial PII proteins have revealed a solution to the signal transduction problem of how to coordinate multiple receptors in response to diverse stimuli yet permit selective control of these receptors under various conditions and allow adaptation of the system as a whole to long-term stimulation.**



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The signal transduction mechanisms responsible for the regulation of development in multicellular organisms probably evolved from the systems controlling metabolism in microorganisms. In microorganisms, development and metabolism are linked at multiple levels. Although they are still largely uncharacterized, global regulatory mechanisms couple flux through different branches of metabolism to provide overall metabolic coordination. These global mechanisms must be operative even when cells are growing optimally in preferred medium, as indicated by the restraint of various metabolic capacities under these conditions. Metabolic stress, such as starvation for an essential nutrient, results in a variety of responses ranging from minor alterations in the cell's proteome to development of a new cell type or spore. Indeed, bacteria of the genus *Myxococcus* form a multicellular tissue as part of development triggered by metabolic stress[1]. Thus, in microorganisms, development can be thought of as a very elaborate form of metabolic regulation.

In this article, we will discuss the remarkable PII signal transduction proteins, which are among the most widely distributed signal transduction proteins in nature, being present in Eukarya, Bacteria and Archaea. In Bacteria and Archaea, PII proteins serve as the central processing unit (CPU) for the integration of antagonistic signals of carbon and nitrogen status, and use this information to control nitrogen assimilation. In microrganisms, nitrogen regulation occurs under all growth conditions. Under favorable conditions, the nitrogen assimilatory capacity of the cell is tightly controlled to coordinate nitrogen assimilation and carbon assimilation. The response to nitrogen starvation involves, as a first step, a minor alteration in the cell's enzymatic capacity. Severe starvation leads to the initiation of development in many microorganisms, with the formation of a specialized cell type that is able to fix atmospheric nitrogen, in some cases in symbiosis with plants. Thus, the response to nitrogen starvation in microorganisms

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