Plasma Membrane Microdomains Act as Concentration Platforms to Facilitate Intoxication by Aerolysin

Laurence Abrami and F. Gisou van der Goot

Department of Biochemistry, University of Geneva, 1211 Geneva 4, Switzerland

Abstract. It has been proposed that the plasma membrane of many cell types contains cholesterol-sphingolipid–rich microdomains. Here, we analyze the role of these microdomains in promoting oligomerization of the bacterial pore-forming toxin aerolysin. Aerolysin binds to cells, via glycosyl phosphatidylinositol-anchored receptors, as a hydrophilic soluble protein that must polymerize into an amphipathic ring-like complex to form a pore. We first show that oligomerization can occur at $>10^5$-fold lower toxin concentration at the surface of living cells than in solution. Our observations indicate that it is not merely the number of receptors on the target cell that is important for toxin sensitivity, but their ability to associate transiently with detergent resistant microdomains. Oligomerization appears to be promoted by the fact that the toxin bound to its glycosyl phosphatidylinositol-anchored receptors, can be recruited into these microdomains, which act as concentration devices.

Key words: aerolysin • cholesterol • microdomains • glycosyl phosphatidylinositol-anchored protein • oligomerization

MANY pathogenic bacteria produce pore-forming toxins as important virulence factors to kill the host cell or to perforate internal cell membranes during bacterial invasion. Similarly, certain cells from the immune system release the membrane attack complex or perforin to perforate the plasma membrane of cells that need to be killed. The modes of action of many pore-forming toxins, of perforin, and of the ninth component of complement are very similar. These proteins are initially soluble and can diffuse towards their target cells to which they bind via specific receptors. The proteins then undergo oligomerization into ring-like amphipathic complexes that are able to insert into membranes and form pores. Although oligomerization is the key step in the channel formation process of all these proteins (for review see Lesieur et al., 1997), it is still poorly understood. Here, we have studied oligomerization of the pore-forming toxin aerolysin at the surface of mammalian cells. This toxin constitutes an attractive model to study pore formation in biological membranes, since its soluble structure has been solved and its in vitro mode of action extensively studied (for review see Rossjohn et al., 1998).

Aerolysin is secreted by the human pathogen *Aeromonas hydrophila*, a member of the *Vibrionaceae* family. The bacterium leads to a variety of infections ranging from gastroenteritis to deep wound infection and septicemia. Strong evidence implicates aerolysin as an important virulence factor produced by the bacterium (Donta and Haddow, 1978; Daily et al., 1981; Kaper et al., 1981; Janda et al., 1984). The toxin is secreted as an inactive soluble precursor, proaerolysin (van der Goot et al., 1993a), that diffuses towards the target cell where it binds to specific receptors. A variety of receptors on different cell types have been identified recently (Gruber et al., 1994; Cowell et al., 1997; Nelson et al., 1997; Abrami et al., 1998b; Diep et al., 1998). These receptors all have the common property of being attached to the plasma membrane via a glycosyl phosphatidylinositol (GPI)[1] anchor. Once bound to the cell surface, the protoxin is activated by proteolytic removal of a COOH-terminal peptide. This can be achieved by cell surface furin (Abrami et al., 1998a), by digestive enzymes, or by proteases produced by the bacterium (Howard and Buckley, 1985). Aerolysin then oligomerizes into a heptameric ring (Wilmsen et al., 1992; Moniatte et al., 1996). This complex is amphipathic and is the membrane insertion competent form (van der Goot et al., 1993b), which penetrates into the membrane of the target cell to form a channel. Therefore, as opposed to the barrel stave model

Address correspondence to Gisou van der Goot, Département de Biochimie, Sciences II, 30 Quai Ernest-Ansermet, 1211 Geneva 4, Switzerland. Tel.: (41) 022 702-6032. Fax: (41) 022 702-6414. E-mail: Gisou.vandergoot@biochem.unige.ch

1. *Abbreviations used in this paper:* β-MCD, β-methyl cyclodextrin; BHK, baby hamster kidney; DIGs, detergent insoluble glycosphingolipid complexes; GMEM, Glasgow minimal essential medium; GPI, glycosyl phosphatidylinositol; IM, incubation medium; PNS, postnuclear supernatant.

for channel formation, oligomerization precedes membrane insertion. Channel formation leads to loss of small molecules and ions triggering in dose-dependent manner signaling cascades (Krause et al., 1998) and selective vacuolation of the endoplasmic reticulum (Abrami et al., 1998b).

As mentioned above, proaerolysin receptors are GPI-anchored proteins. These proteins, which are not transmembrane, but are anchored to the membrane via a lipid moiety (Kinoshita et al., 1997), have attracted much attention lately, not only because of their role in signaling events (Jones and Varela-Nieto, 1998), but also due to their possible role in apical targeting in polarized epithelial cells and their association with cholesterol glycosphingolipid-rich microdomains, also called lipid rafts (for review see Parton and Simons, 1995; Edidin, 1997; Harder and Simons, 1997; Simons and Ikonen, 1997; Brown, 1998; Hooper, 1998; Brown and London, 1998; Jacobson and Dietrich, 1999). It was the identification of lipid fractions that were insoluble in nonionic detergents, such as Triton X-100 at 4°C, that led to the proposal that these domains form lipid microdomains within the plasma membrane (see above reviews). However the actual existence of lipid rafts in living cells has been a matter of debate (Sheets et al., 1997; Friedrichson and Kurzchalia, 1998; Harder et al., 1998; Kenworthy and Edidin, 1998; Varma and Mayor, 1998), and the issue has not yet been definitively solved (for review see Jacobson and Dietrich, 1999).

Previously, we have shown that receptor-bound proaerolysin is highly enriched in detergent insoluble complexes (Abrami et al., 1998b). The aim of the present work was to address the importance of the distribution of aerolysin receptors at the plasma membrane for the oligomerization process. Aerolysin provides an ideal model system for these studies because the aerolysin heptamer is SDS resistant and can, therefore, readily be visualized by SDS-PAGE. In addition, cholesterol is not absolutely essential for aerolysin binding and channel formation (van der Goot et al., 1993b), thereby allowing manipulation of cellular cholesterol to perturb the structure of microdomains. Here, we first show that the aerolysin heptamer, in addition to proaerolysin, is present in the detergent in soluble glycosphingolipid complexes (DIGs). Our data show that disruption of the microdomains prevents toxin clustering at the cell surface and dramatically inhibits oligomerization. Based on these data, we propose that cholesterol-rich microdomains act as specialized concentration devices on the plasma membrane. Finally, evidence is provided that processing of proaerolysin into aerolysin and oligomerization are events that occur in topologically distinct areas of the plasma membrane, highlighting the dynamic behavior of GPI-anchored proteins at the cell surface.

Materials and Methods

Cells and Materials

Monolayers of baby hamster kidney (BHK) cells were grown and maintained as described (Gruenberg and Howell, 1989) in Glasgow minimal essential medium (GMEM; Sigma Chemical Co.) supplemented with 5% FCS, 2 mM L-glutamine under standard tissue culture conditions. Triton X-100 Ultra Pure was purchased from Pierce Chemical Co., BSA from Biomol, and saponin, β-methyl cyclodextrin (β-MCD), compactin, trypsin, and trypsin/chymotrypsin inhibitor from Sigma Chemical Co. Antibodies against the human transferrin receptor and proaerolysin were gifts from I. Throwbridge (The Salk Institute for Biological Studies, San Diego, CA) and J.T. Buckley (University of Victoria, British Columbia, Canada), respectively. mAbs against caveolin-1 were purchased from Transduction Laboratories.

Treatment of Cells with Cholesterol-affecting Drugs

BHK cells were grown, as described, for 16 h. Before the addition of the toxin, the cells were washed three times with PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 μg/ml of trypsin/chymotrypsin inhibitor (PBS^{2+}), and treated with either 0.4% saponin in PBS^{2+} for 1 h at 4°C or with 10 mM β-MCD for 1 h at 37°C in incubation medium (IM) containing GMEM buffered with Hepes, pH 7.4, and 1 μg/ml of trypsin/chymotrypsin inhibitor.

Proaerolysin Purification, Iodination, and Activation

Proaerolysin was purified as described previously (Buckley, 1990). Concentrations were determined by measuring the OD at 280 nm, considering that a 1 mg/ml sample has an OD of 2.5 (van der Goot et al., 1994a). Proaerolysin was labeled with ^{125}I using iodogen reagent (Pierce Chemical Co.) according to the manufacturer's recommendations. The radiolabeled toxin was separated from the free iodine by gel filtration on a PD10-G25 column (Pharmacia Biotech, Inc.) equilibrated with 150 mM NaCl, 20 mM Hepes, pH 7.4. We consistently obtained a specific activity of $\sim 2.10^6$ cpm/μg of proaerolysin. Radiolabeled proaerolysin ran as a single band on an SDS gel.

To activate proaerolysin into aerolysin in vitro, the protoxin was treated with trypsin (1:20; mol:mol ratio) for 10 min at room temperature. The reaction was stopped by addition of a 10-fold excess of trypsin/chymotrypsin inhibitor.

Proaerolysin Binding and Oligomerization on BHK Cells

Confluent monolayers of BHK cells were washed three times for 5 min with ice-cold PBS^{2+}. Cells were then incubated at 4°C with ^{125}I-proaerolysin in IM. Cells were then washed three times for 10 min with PBS^{2+} at 4°C and further incubated for various times at 37°C. Cells were then rinsed three times with PBS^{2+} at 4°C, scraped from the dish, and collected by centrifugation at 1,500 rpm for 5 min. A Triton X-100 insoluble fraction was prepared as described below or a postnuclear supernatant (PNS) was prepared as follows. Cells were gently homogenized in 250 mM sucrose, 3 mM imidazole, pH 7.4, by passage through a 22G injection needle. The PNS was obtained by centrifugation (2,500 rpm) and analyzed for the presence of proaerolysin and aerolysin by SDS-PAGE, followed by autoradiography.

Triton X-100 Insolubility and Preparation of DIGs

The detergent extraction was performed as described previously (Brown and Rose, 1992; Fra et al., 1994; Abrami et al., 1998b). BHK cells were treated with ^{125}I-proaerolysin and harvested as described above, and resuspended in 0.5 ml of cold buffer containing 25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, as well as Complete, a cocktail of protease inhibitors (Boehringer Mannheim Corp.). Membranes were solubilized by rotary shaking at 4°C for 30 min. The detergent insoluble fractions were obtained either by high-speed centrifugation (30 min, 4°C, at 55,000 rpm in a TLS55 Beckman rotor) or purified on a sucrose gradient as follows. The solubilized cell lysate sample was adjusted to 41% sucrose (in 10 mM Tris-HCl, pH 7.4) in a SW40 Beckman tube, overlaid with 8.5 ml of 35% sucrose, topped up with 16% sucrose, and centrifuged for 18 h at 35,000 rpm at 4°C. The initial load (1 ml), as well as 12 fractions of 1 ml, were collected. Each of the fractions were counted in a Packard auto-γ scintillation spectrometer and/or precipitated with 6% trichloroacetic acid in the presence of 375 μg of sodium deoxycholate as a carrier. Each fraction was analyzed by SDS-PAGE, followed by autoradiography or Western blot analysis.

Immunofluorescence

BHK cells were grown for 16 h on 12 × 12-mm glass coverslips in GMEM medium complemented with 2.5% FCS. Cells were left untreated or treated with saponin or β-MCD, as described above. After three washes

with PBS^{2+} at 4°C, cells were incubated for 1 h at 4°C with 2 nM of proaerolysin, then washed three times for 10 min with PBS^{2+} complemented with 0.5% BSA at 4°C, further incubated for 1 h at 4°C with mAbs against proaerolysin (1:40; Abrami et al., 1998b), and then washed briefly at 4°C in PBS^{2+}–BSA. Further cross-linking with FITC secondary antibodies (1:50) was performed at 4°C for 1 h. After washing, the cells were fixed first in 3% paraformaldehyde in PBS for 4 min at 8°C and then in methanol for 5 min at −20°C. Cells were visualized using a Zeiss Axiophot fluorescence microscope equipped with a cooled CCD camera (Princeton Instruments), controlled by a Power Macintosh. The IPLab Spectrum 3.1 software (Signal Analytics Corp.) was used for data acquisition.

Analytical Techniques and Proaerolysin Overlays

Potassium efflux measurements were performed by flame photometry using a Philips PYE UNICAM SP9 atomic absorption spectrophotometer as described previously (Abrami et al., 1998a,b). Protein concentrations of cellular fractions were determined with bicinchoninic acid (BCA; Pierce Chemical Co.). Proaerolysin overlays, to detect proaerolysin receptors, were performed as described previously (Abrami et al., 1998b). In brief, membrane fractions were run on a 12.5% SDS gel (Laemmli, 1970). The gel was incubated in 50 mM Tris-HCl, pH 7.4, and 20% glycerol for 15 min. Proteins were then blotted onto a nitrocellulose membrane for 15 h at 100 mA in the cold using a BioRad wet transfer chamber with a transfer buffer containing 10 mM $NaHCO_3$ and 3 mM Na_2CO_3, pH 9.8. The nitrocellulose membrane was incubated in a binding buffer containing 50 mM NaH_2PO_4, pH 7.5, and 0.3% Tween-20 for 20 min followed by a 2 h incubation in the presence of 1.4 nM ^{125}I-proaerolysin diluted in the same buffer. The membrane was then washed six times for 5 min with toxin-free binding buffer. Binding of proaerolysin was revealed by autoradiography using BioMax films (Kodak). When performing Western blot analysis, HRP-labeled secondary antibodies were revealed using enhanced chemiluminescence (Pierce Chemical Co.). Total cholesterol contents of cells were determined using a colorimetric method following the instructions of the manufacturer (Boehringer Mannheim Corp.).

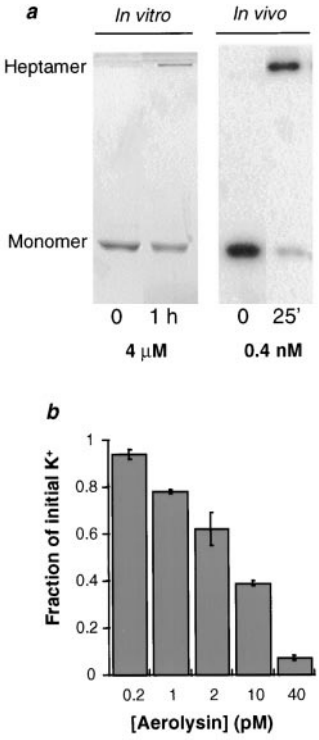

Figure 1. Oligomerization of aerolysin is more efficient in vivo than in vitro. a, To follow oligomerization in vitro, proaerolysin at a concentration of 4 μM was activated with trypsin as described in Materials and Methods. The sample was then incubated at 37°C for 1 h and analyzed by SDS-PAGE, followed by Coomassie staining. The aerolysin heptamer (333 kD) is SDS resistant and can be visualized at the top of the gel. For the in vivo oligomerization experiment, ^{125}I-proaerolysin at a concentration of 0.4 nM was activated in solution with trypsin and added to BHK cells at 4°C for 1 h. Cells were then washed and incubated for 25 min at 37°C. A PNS was prepared and analyzed by SDS-PAGE, followed by autoradiography. b, To measure potassium efflux from BHK cells at different toxin concentrations, cells were incubated with aerolysin for 1 h at 37°C and the cellular potassium contents were determined by flame photometry. Experiments were done in triplicate and the SDs were calculated.

Results

Oligomerization Is Dramatically Enhanced at the Surface of Target Cells

Aerolysin is able to form heptamers in solution in the absence of membranes (van der Goot et al., 1994b; Parker et al., 1996; Rossjohn et al., 1998). However, this process is not very efficient. As can be seen in Fig. 1 a, at a toxin concentration of 4 μM, <50% of the toxin had oligomerized, even after 1 h incubation at 37°C. In marked contrast, oligomerization occurred efficiently on the surface of target cells, such as BHK cells. When BHK cells were incubated with a 10^4-fold lower concentration of toxin (0.4 nM), 25 min at 37°C was sufficient to observe almost complete oligomerization (Fig. 1 a, in vivo). Oligomerization and channel formation occurred at even lower doses, such as 1 pM of aerolysin, as witnessed by measuring the intracellular potassium concentration 1 h after toxin addition (Fig. 1 b). Thus, oligomerization at the cell surface can occur at bulk toxin concentrations 10^5–10^6-fold lower than those required to detect oligomerization in solution.

All Forms of the Toxin Are Enriched in Triton X-100 Insoluble Complexes

Previous observations indicated that the toxin was associated with DIGs of the plasma membrane (Abrami et al., 1998b). Here we investigated whether all forms of the toxin, i.e., not only proaerolysin, but also mature aerolysin and the heptamer, were enriched in DIGs. ^{125}I-Proaerolysin was bound to cells at 4°C. Cells were then incubated either with proaerolysin at 4°C or with aerolysin (obtained by in vitro activation with trypsin) at 4°C followed by 10 min at 37°C to allow heptamerization (Abrami et al., 1998a,b). Cells were subsequently solubilized in Triton X-100 and DIGs were purified on a sucrose flotation gradient as described in Materials and Methods. As shown in Fig. 2, not only proaerolysin was highly enriched in the low-density DIGs in agreement with our previous observations (Abrami et al., 1998b), but also mature aerolysin and the heptamer.

We next quantified the fraction of the total toxin that was associated with detergent insoluble domains as a function of the incubation time at 37°C. As previously shown (Abrami et al., 1998a), 45% of the toxin initially bound to the cells at 4°C was released into the medium upon incubation at 37°C, the remaining 65%, however, was stably bound to the cells (Fig. 3 a). We then investigated whether the distribution of the cell-bound toxin would change with time. In these experiments, toxin-treated cells were incubated for different times at 37°C, then solubilized in cold Triton X-100 and submitted to a high-speed centrifugation. The amounts of toxin in the detergent insoluble pellet and in the solubilized fraction were determined. As can be seen in Fig. 3 b, the amount of toxin associated with detergent-resistant membranes increased with time, consistent with the notion that the toxin was being recruited into these microdomains, presumably because of oligomerization.

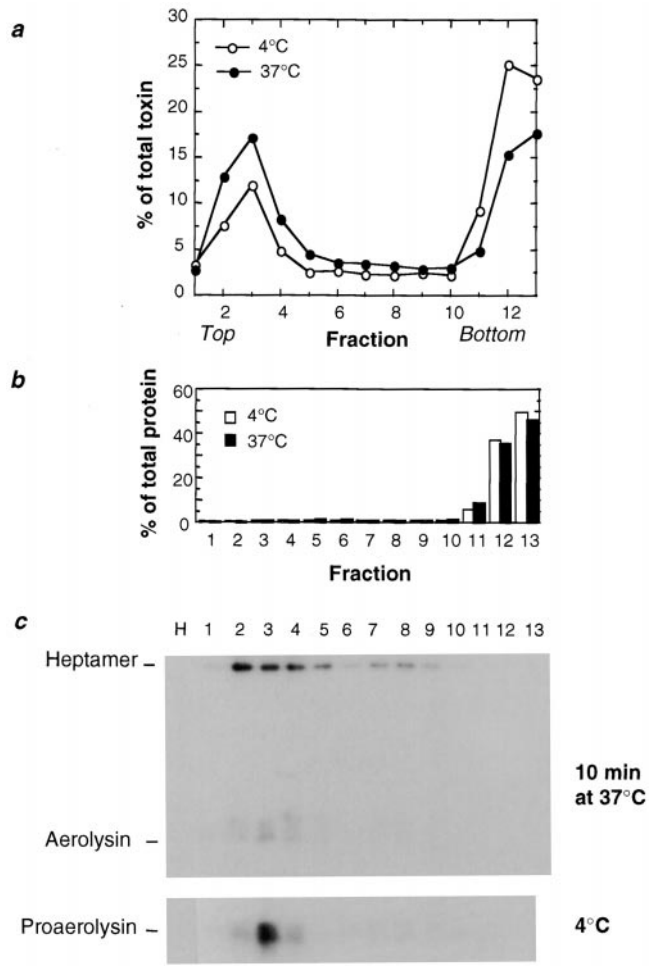

Figure 2. Proaerolysin, aerolysin, and heptameric aerolysin are enriched in Triton X-100 insoluble microdomains. ^{125}I-proaerolysin or trypsin-activated ^{125}I-aerolysin (0.4 nM) were bound to cells at 4°C for 1 h. Cells were then washed and directly solubilized in Triton X-100 (proaerolysin-treated cells) or first incubated for 10 min at 37°C (aerolysin-treated cells) to allow oligomerization, and then solubilized in Triton X-100. DIGs were purified on sucrose density gradients as described in Materials and Methods. The initial 1-ml Triton X-100 lysat was loaded at the bottom of the tube corresponding to fraction 13. The amount of radioactivity (a) associated with each fraction of the gradient was counted and the protein concentration of each fraction was determined (b). The enrichment profile of toxin (c) was analyzed by SDS-PAGE, followed by autoradiography by loading the same amount of protein (20 μg) in each lane. All forms of the toxin were enriched in the low-density fractions.

Effects of Cholesterol Interacting Drugs on the Distribution of Proaerolysin

Since all forms of aerolysin were found in DIGs, we investigated whether disruption of these microdomains had any influence on the protoxin cleavage and oligomerization processes. The integrity of microdomains has been shown to depend on cholesterol (for review see Brown, 1998; Harder et al., 1998; Rietveld and Simons, 1998). Moreover, cholesterol has been shown to be implicated in the detergent insolubility of GPI-anchored proteins (Cerneus et al., 1993; Hannan et al., 1993; Hanada et al., 1995; Scheiffele et al., 1997; Harder et al., 1998; Schroeder et al., 1998). We therefore analyzed the effect of cholesterol-affecting drugs on the association of proaerolysin with detergent insoluble membrane domains. It is important to note, that, in contrast to what is observed with pore-forming toxins, such as streptolysin O and other members of the thiol-activated toxin family, no requirement for cholesterol has ever been observed for channel formation by aerolysin in artificial phospholipid membranes, suggesting that cholesterol does not play any crucial structural role in aerolysin channel formation and can, therefore, be manipulated in the present experiments.

Two drugs were tested: β-MCD and saponin. β-MCD is an effective extracellular cholesterol acceptor that can extract cholesterol from membranes (Kilsdonk et al., 1995; Neufeld et al., 1996). Saponin, in contrast, binds to cholesterol and sequesters it away from other interactions, but does not extract it from the membrane (Elias et al., 1978; Schroeder et al., 1998). Under the experimental conditions used, 50 ± 2% (n = 4) of total cellular cholesterol was removed by β-MCD, whereas the cholesterol content of saponin-treated cells was the same as that of control cells, as expected. After drug treatment, cells were incubated with ^{125}I-proaerolysin for 1 h at 4°C. Neither drug prevented

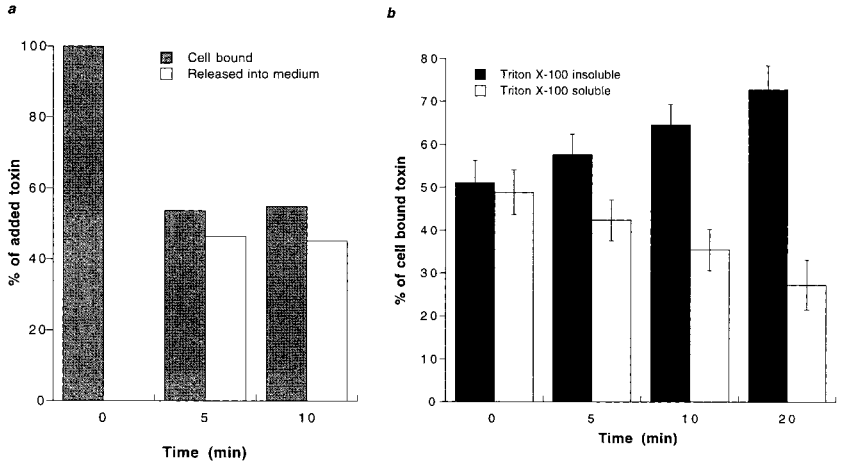

Figure 3. The amount of toxin associated with detergent resistant membranes increases with time. BHK monolayers were incubated with ^{125}I-proaerolysin (0.4 nM) for 1 h at 4°C, then extensively washed and further incubated for different times at 37°C. a, The amount of radioactivity associated with the cells and released into the medium was determined and expressed as a percent of the total. b, Cells were solubilized in Triton X-100 and submitted to a high-speed centrifugation. The total radioactivity in the detergent insoluble pellets and the detergent soluble supernatants were determined and expressed as a percent of the total. Error bars represent the mean of four experiments.

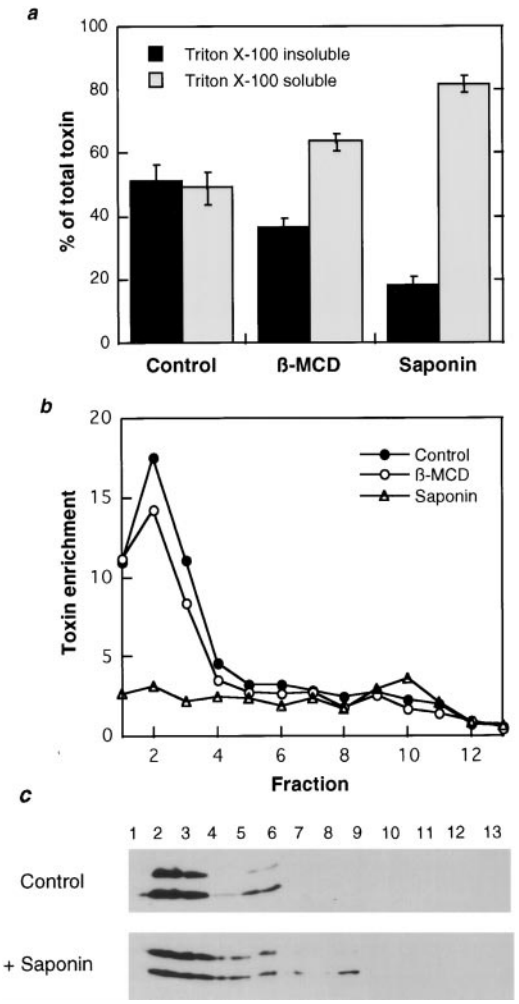

Figure 4. Effect of cholesterol-affecting drugs on the distribution of proaerolysin. BHK monolayers were treated or not with β-MCD (10 mM in IM at 37°C for 1 h) or saponin (0.4% in PBS^{2+} at 4°C for 1 h). Cells were then incubated with ^{125}I-proaerolysin (0.4 nM) for 1 h at 4°C and solubilized in Triton X-100. a, To separate the detergent insoluble complexes from the solubilized material, the samples were centrifuged at high speed. The total radioactivity in the pellets and the supernatants were determined and expressed as a percent of the total. b, DIGs were prepared by sucrose density gradient. The initial 1-ml Triton X-100 lysat was loaded at the bottom of the tube corresponding to fraction 13. The amount of toxin in each fraction was counted. The protein content of each fraction was determined by BCA protein assay. More than 85% of the total protein content was found in fractions 11 to 13 (results not shown). The enrichment factors were calculated as follows: (counts in fraction x ÷ protein content of fraction x) ÷ (total counts on gradient ÷ total protein on gradient). The results are the mean of at least two experiments (control, $n = 9$; saponin, $n = 5$; β-MCD, $n = 2$). c, DIGs obtained from saponin-treated or control cells, as in b, were analyzed by SDS-PAGE, followed by Western blotting to detect the presence of the caveolar marker, caveolin-1. The same amounts of protein (20 μg) were loaded in each lane to show enrichment.

binding of the toxin to the cells (see Figs. 6–8). Toxin-treated cells were then solubilized in cold Triton X-100, submitted to a high-speed centrifugation, and the amounts of toxin in the detergent insoluble pellet and in the solubilized fraction were determined. As shown in Fig. 4 a, β-MCD led to a mild redistribution of proaerolysin to the detergent soluble fraction. However, after saponin treatment, >80% of cell bound proaerolysin was associated with the detergent soluble fraction. Similar observations were made when purifying DIGs by sucrose density centrifugation from proaerolysin-treated cells. The distribution of proaerolysin along the gradient after β-MCD treatment (results not shown) was similar to that observed for control cells (Fig. 2 a). Therefore, the toxin was still highly enriched in DIGs after β-MCD treatment, but this was no longer the case after saponin treatment (Fig. 4 b). However, saponin did not disrupt all DIGs since the caveolar marker caveolin-1 was still highly enriched in the light density detergent insoluble fractions of the gradient, as also observed for control cells (Fig. 4 c) and cells treated with β-MCD (results not shown).

Previously, we have shown that receptor bound proaerolysin can be clustered using antitoxin antibodies, and that clustering leads to a characteristic punctate distribution by immunofluorescence (Abrami et al., 1998b). Within these clusters, the toxin was found to fully colocalize with antibody cross-linked alkaline phosphatase and partially with caveolin-1 (Abrami et al., 1998b). Similar antibody-induced clustering has been observed for a variety of GPI-anchored proteins (for review see Brown and London, 1998). We have investigated whether treatments with β-MCD or saponin would affect this antibody-induced clustering capacity. The staining pattern found on β-MCD–treated cells (Fig. 5 b) was similar to that observed on control cells. In contrast, a diffuse staining was observed when cells were treated with saponin (Fig. 5 c) indicating that, under these conditions, even a sandwich of primary and secondary antibodies, before cell fixation, could not induce clustering of proaerolysin bound to its receptor.

The above experiments indicate that cholesterol removal with β-MCD did not alter the ability of proaerolysin to associate with microdomains, but that cholesterol clustering with saponin led to a redistribution of the receptor-bound toxin over the entire plasma membrane. These experiments also suggest that β-MCD and saponin affect different pools of cholesterol within the plasma membrane.

β-MCD Leads to an Acceleration of Protoxin Processing

Despite the lack of effect of β-MCD on steady state proaerolysin distribution, we analyzed the effect of the drug on the cleavage and oligomerization processes. Interestingly, we found that β-MCD treatment led to a dramatic acceleration of the proteolytic processing of proaerolysin into aerolysin (Fig. 6 a). In contrast, there was no effect on the oligomerization kinetics, as can be seen when treating cells with aerolysin that had been activated in vitro to bypass the cell surface activation step (Abrami et al., 1998a; Fig. 6 b). The same observation was made when β-MCD was present throughout the experiment (including incubation with the toxin at 37°C) in addition to the cholesterol synthesis inhibitor compactin. Our interpretation of the effects of β-MCD is that the drug fa-

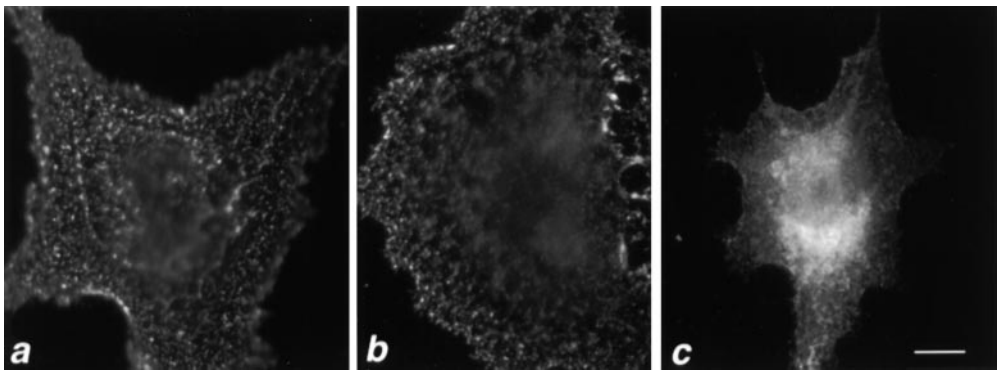

Figure 5. Antibody cross-linking of receptor bound proaerolysin at the cell surface. BHK cells were treated or not with β-MCD (10 mM in IM at 37°C for 1 h) or saponin (0.4% at 4°C for 1 h), then incubated consecutively at 4°C with proaerolysin (2 nM) for 1 h, with anti-proaerolysin mAbs for 1 h, and finally with FITC-labeled secondary antibodies for 1 h. Cells were then fixed first in 3% paraformaldehyde in PBS for 4 min at 8°C and then in methanol for 5 min at −20°C. The punctate pattern observed in β-MCD cells (b) was similar to that observed in control cells (a), but could not be found in any of the saponin-treated cells (c). Similar patterns were observed when fixing cells with paraformaldehyde (5 min at 4°C, followed by 20 min at room temperature) only, even after treatment with 0.2% or 0.4% saponin for 30 min at 4°C. The staining observed in the nuclear region in saponin-treated cells is due to background staining of the antibody and can also be seen when cells have not been treated with the toxin (results not shown). This staining is absent in control and β-MCD–treated cells because the cells are not permeabilized during antibody treatment. Bar, 10.5 μm.

vored the interaction between the enzyme and protoxin because the drug affected regions of the plasma membrane that contain the proaerolysin-processing enzyme, but does not significantly affect the microdomains enriched in GPI-anchored proteins (Figs. 4 b and 5 b). Previously, we have shown that processing of proaerolysin at the cell surface is primarily performed by the endoprotease furin (Seidah and Chretien, 1997; Molloy et al., 1999) in both CHO (Abrami et al., 1998a) and BHK cells (our unpublished results). We could not detect any in vitro processing of proaerolysin bound to DIGs, suggesting that furin is not present to any significant extent in microdomains (results not shown).

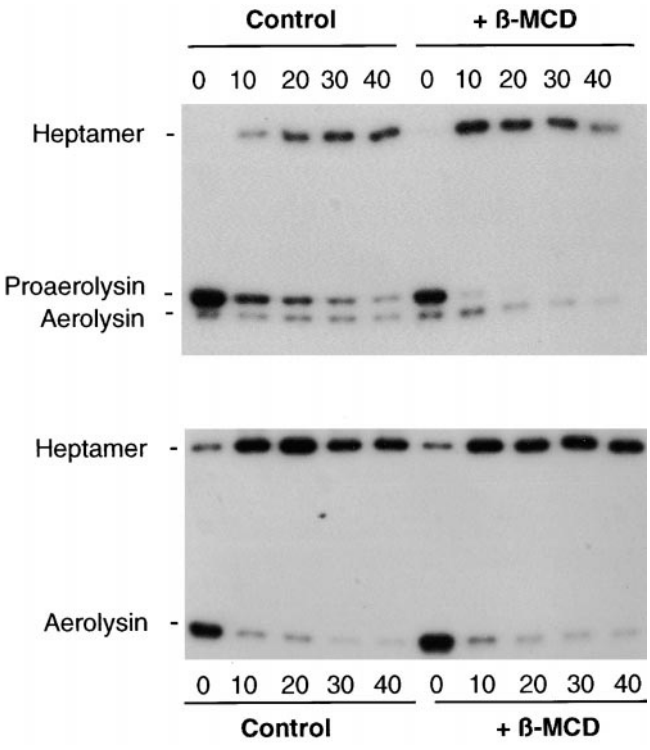

Figure 6. β-MCD does not affect oligomerization, but dramatically accelerates protoxin processing. BHK monolayers were treated with β-MCD (10 mM in IM at 37°C for 1 h), then incubated with either ^{125}I-proaerolysin (a) or trypsin-activated ^{125}I-aerolysin (b; 0.4 nM) for 1 h at 4°C and subsequently incubated at 37°C for various times (indicated in minutes on the figure). PNSs were prepared and analyzed by SDS-PAGE, followed by autoradiography (20 μg of protein were loaded per lane). After β-MCD treatment, conversion of proaerolysin into aerolysin was dramatically accelerated.

Saponin Treatment Leads to the Inhibition of Aerolysin Oligomerization

As shown in Fig. 4 a, saponin led to a dramatic redistribution of proaerolysin to the detergent soluble fraction of Triton X-100–treated cells. This treatment, however, did not lead to solubilization of the plasma membrane since the total phospholipid content of saponin-treated cells was the same as that of control cells, as measured using the Fiske-Subbarow phosphore determination method (results not shown). As mentioned above, the total cholesterol content was similar to that of control cells. Also, the plasma membrane distribution of the transferrin receptor was not affected by the treatment (results not shown). Finally, toxin receptors were not solubilized by this treatment since binding of aerolysin was not affected, and the same amount of receptors could be detected in control and saponin-treated cells using our previously established toxin overlay assay (Fig. 7 a). This overlay assay led to the identification of various GPI-anchored proaerolysin binding proteins, including a 130- and a 80-kD protein, the 80-kD protein being apparently the most abundant receptor (Abrami et al., 1998b).

The redistribution of proaerolysin to the detergent soluble fractions upon saponin treatment was paralleled by a redistribution of the proaerolysin receptors (Fig. 7 b), indicating that solubilization of the toxin is not due to an effect of the drug on the toxin-receptor interaction. Previously, it has been shown that saponin treatment led to the Triton X-100 solubilization of other GPI-anchored proteins (Cerneus et al., 1993; Hanada et al., 1993, 1995).

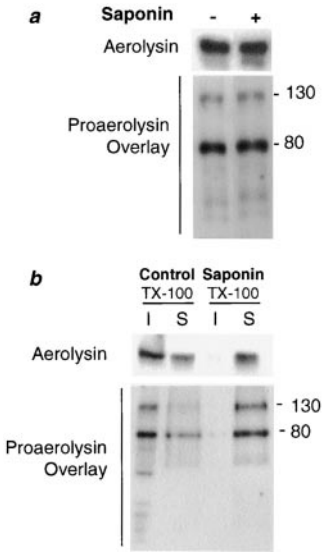

Figure 7. Effects of saponin treatment on aerolysin binding and receptor distribution. a, BHK monolayers were left untreated or treated with saponin (0.4% at 4°C for 1 h) and then incubated with ^{125}I-proaerolysin (0.4 nM) for 1 h at 4°C. PNSs were prepared and analyzed by SDS-PAGE, followed by autoradiography, to detect bound toxin; or by toxin overlay, followed by autoradiography, to detect the proaerolysin receptors (20 μg of protein were loaded per lane). All bands detected by toxin overlay are GPI-anchored proteins and can no longer be detected when cells have been treated with the phosphatidyl inositol-specific phospholipase C (Abrami et al., 1998b). b, BHK monolayers were left untreated or treated with saponin, as in a. Cells were scrapped, pelleted, and resuspended in 1% Triton X-100 at 4°C. Membranes were solubilized by rotary shaking at 4°C for 30 min. The detergent insoluble fractions were obtained by high-speed centrifugation (30 min at 4°C at 55,000 rpm). Detergent insoluble (I) and soluble (S) fractions were analyzed by SDS-PAGE, followed by autoradiography, to detect bound toxin; or by toxin overlay, followed by autoradiography, to detect the proaerolysin receptors. One tenth of each fraction was loaded per lane (corresponding to ~80 μg of the Triton X-100 soluble fraction) to analyze the recovery of the toxin and its receptors.

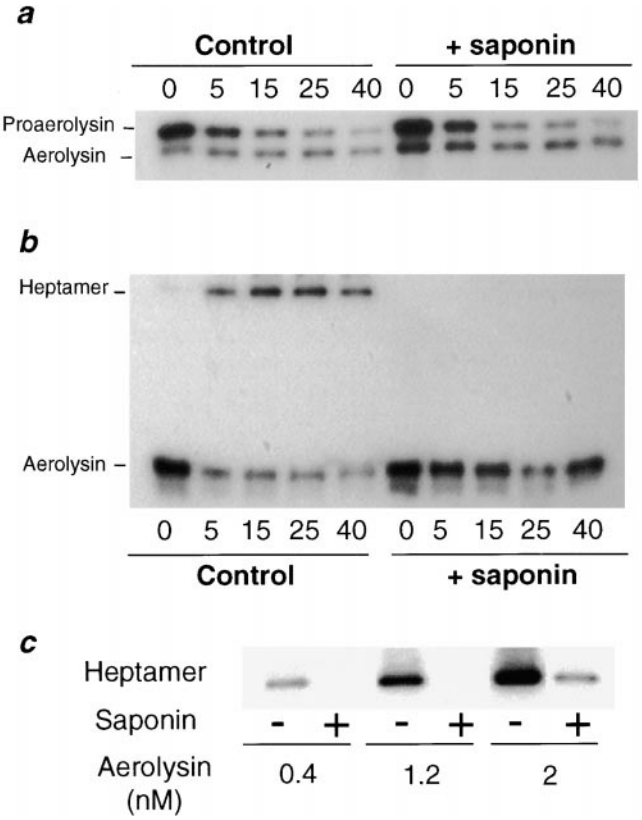

Figure 8. Saponin inhibits the kinetics of aerolysin oligomerization. BHK monolayers were left untreated or treated with saponin (0.4% at 4°C for 1 h). Cells were then incubated with ^{125}I-proaerolysin (0.4 nM; a) or in vitro trypsin-activated ^{125}I-aerolysin (0.4 nM; b) for 1 h at 4°C, washed, and further incubated for various times at 37°C (indicated in minutes on the figure). PNSs were prepared and analyzed by SDS-PAGE, followed by autoradiography (20 μg of protein were loaded per lane). After prolonged incubation at 37°C, a slight loss of cell-bound toxin is observed in saponin-treated cells for reasons that remain to be established. c, BHK monolayers were left untreated or treated with saponin as in a, and then incubated for 1 h at 4°C with different concentrations of in vitro trypsin-activated ^{125}I-aerolysin. At all concentrations tested, binding of aerolysin to the cells was specific. Cells were then washed and further incubated for 25 min at 37°C. PNSs were prepared and analyzed by SDS-PAGE, followed by autoradiography.

We next tested the effects of saponin both on cleavage of proaerolysin by furin and on heptamerization kinetics. Cells were treated with saponin at 4°C, then incubated in the presence of proaerolysin at 4°C, and further incubated for various times at 37°C. As shown in Fig. 8 a, the kinetics of cleavage were not affected by saponin, in contrast to what we observed with β-MCD (Fig. 6 a). This observation indicates that overall cell surface dynamics were not significantly affected by saponin-treatment. In contrast, oligomerization was dramatically inhibited, as can clearly be seen when treating cells with aerolysin activated in vitro with trypsin (Fig. 8 b). This was not due to some effect of saponin on the toxin itself, since saponin had no effect on oligomerization kinetics in vitro (results not shown). Similar observations were made on other cell types, such as CHO cells or the polarized human intestinal cell line, CaCoII (results not shown). Saponin prevented clustering of cell surface-bound toxin and dramatically inhibited the oligomerization process.

Since the toxin was no longer found in microdomains and showed uniform cell surface labeling, yet remained bound to its receptor, it is likely that saponin caused the receptors and their bound toxin molecules to become evenly distributed in the plane of the plasma membrane by preventing any clustering. The absence of clustering due to saponin would reduce the probability of encounter of toxin monomers and thereby the efficiency of oligomerization. If so, one might expect to, at least to some extent, counteract the action of saponin by increasing the amount of cell-bound toxin, hence the probability of toxin monomer–monomer interactions. As can be seen in Fig. 8 c, this is indeed the case. When higher toxin concentrations were added to the saponin-treated cells, oligomerization did occur. The efficiency of the process, however, remained much lower than on control cells, further strengthening the importance of toxin concentration within microdomains on the oligomerization process.

Discussion

The present work illustrates the surface dynamics of pro-

aerolysin upon binding to its GPI-anchored receptor on target cells. Proaerolysin must first be proteolytically processed to become active. The toxin then oligomerizes into a heptameric ring, a step that precedes membrane insertion and channel formation (van der Goot et al., 1993b). Using two cholesterol-perturbing drugs, we could selectively affect the kinetics of processing or of oligomerization, suggesting that the two events occur in topologically distinct areas of the plasma membrane. Processing appeared to occur in the phosphoglyceride region, whereas oligomerization was most efficient in cholesterol-rich microdomains, which act as concentration devices at the cell surface.

Proaerolysin Processing

Previously, we have shown that processing of proaerolysin at the cell surface is primarily performed by the endoprotease furin (Abrami et al., 1998a). Since furin contains a canonical Tyr-based and a dileucine-like internalization signal in its cytoplasmic tail, it is thought to be internalized via clathrin-coated pits (for review see Seidah and Chretien, 1997; Molloy et al., 1999). Furin is therefore expected to be in the phosphoglyceride region of the membrane and not in cholesterol-rich microdomains. In agreement, we could not detect any proaerolysin converting activity in purified microdomains.

We found that β-MCD treatment of cells, which led to the removal of ~50% of total cholesterol, accelerated the processing of proaerolysin, presumably by increasing the lateral mobility of both furin and receptor-bound proaerolysin. In contrast, the drug did not affect the ability of the toxin to associate with detergent resistant microdomains. Our interpretation is that β-MCD extracted cholesterol from a pool that is not involved in the association of GPI-anchored proteins with microdomains. Cholesterol is indeed not only found in raft-like microdomains, but also in the fluid phosphoglyceride regions (Chong, 1994), and has also been predicted to accumulate at the edge of microdomains (Cruzeiro-Hansson et al., 1989; Gil et al., 1998). In agreement with our observations, Ilangumaran and Hoessli (1998) suggested that β-MCD only affects cholesterol surrounding and outside of GPI-containing microdomains. However, effects of β-MCD on the distribution of GPI-anchored proteins have been observed also (Friedrichson and Kurzchalia, 1998; Harder et al., 1998). These differences in the observed effects of β-MCD might be due to differences in cell lines and/or in culture conditions.

Aerolysin Oligomerization

The next crucial step in the channel formation process of aerolysin is oligomerization. Previously, we have shown that in vitro, in the absence of membranes, at physiological salt concentrations and temperature, heptamers can only form when the toxin concentration is higher than 1 μM (van der Goot et al., 1992). On target cells, however, oligomerization was found to occur even when adding a 10^5-fold lower aerolysin concentration. Several factors might contribute to this increased efficiency of oligomerization.

Binding of the toxin to its receptors directs it to its target cell. Moreover, since the estimated binding dissociation constant (K_d) is low (Abrami et al., 1998b), it can be expected that binding also concentrates the toxin. Indeed, as discussed first by Adam and Delbrück (1968) and later by McLaughlin and Aderem (1995), membrane binding reduces the dimensionality from three to two. Using the Guggenheim model of a surface (McLaughlin and Aderem, 1995), we calculated that toxin binding to its receptor leads to an increase in concentration by a factor of ~1,500 (see Appendix). Although this estimate may be approximate, it strongly suggests that binding cannot solely account for increased efficiency of oligomerization observed at the cell surface, as compared with in solution. Additional factors are likely to contribute. Here, we show that cholesterol-rich microdomains play an important role. Due to the long and saturated acyl chains of the GPI-moiety, GPI-anchored proteins have the capacity to associate transiently with lipid rafts (Brown and London, 1998). We found that on cells that had been treated with the cholesterol-binding drug saponin, proaerolysin lost its capacity to associate with microdomains (Fig. 4) and to cluster upon cross-linking with antibodies (Fig. 5 c). Concomitantly, saponin led to a dramatic inhibition of aerolysin oligomerization, without altering toxin activation kinetics, suggesting that the concentration threshold required for heptamer formation could no longer be reached, even locally. Oligomerization could, however, be forced when adding higher amounts of toxin to saponin-treated cells, but remained far less efficient, as expected.

These results show that microdomains act as concentration platforms at the cell surface, due to their ability to recruit GPI-anchored proteins, and that aerolysin has hijacked this device to suit its own purpose. When the same number of toxin receptors were dispersed at the plasma membrane and prevented from clustering by saponin treatment, oligomerization either did not occur at all or kinetics were dramatically inhibited, depending on the toxin concentration. The corollary of these observations is that having surface receptors that can cluster renders cells more sensitive to low doses of toxin. Recently, it has been proposed by Bray et al. (1998) that receptor clustering could be a cellular mechanism to control the sensitivity of a cell to a given ligand. Backed-up by a mathematical model, they proposed that the sensitivity and response range of chemotatic bacteria to attractants depend on the clustering of the chemotatic receptors on the cell surface (Bray et al., 1998). The present findings demonstrate experimentally that this proposal is correct for pore-forming toxins, such as aerolysin.

We believe the importance of microdomains for efficient oligomerization will extend to other pore-forming proteins, both toxins and proteins, from the immune response (Fivaz et al., 1999). *Vibrio Cholera* cytolysin has been shown to require both cholesterol and sphingolipids to form channels in vitro (Zitzer et al., 1999). Lysenin, a pore-forming toxin for earthworms, has been shown to bind to sphingomyelin (Lange et al., 1997; Yamaji et al., 1998). Both these toxins, which require oligomerization for channel formation, are likely to bind to cholesterol-sphingolipid–rich microdomains on living cells. A major class of pore-forming toxins is composed of the so-called thiol-activated toxins, which include streptolysin O, lysteriolysin O, and perfringolysin O (Tweten, 1995). Members

of this family require cholesterol to form pores (Bhakdi et al., 1996), even though cholesterol might not be the receptor, as initially thought (Jacobs et al., 1998). Cholesterol binding to these toxins probably triggers a conformational change necessary for membrane insertion. We would like to speculate that cholesterol, in addition, targets thiol-activated toxins to microdomains to increase in local toxin concentration to promote oligomerization. Clustering of thiol-activated toxins is probably even more important than for aerolysin, since the pores contain an average of 50 monomers (Lesieur et al., 1997). Similarly, the pores formed by the ninth component of complement contain ~20 monomers (Peitsch and Tschopp, 1991).

In the present work, we describe the journey of proaerolysin on the plasma membrane of the target cell and how the toxin makes use of the peculiar properties of its GPI-anchored receptors to optimize its toxic action. Importantly, GPI-anchored proteins can be found both scattered in the phosphoglyceride region of the plasma membrane and clustered in the microdomains. Indeed, only ~35% of the GPI-anchored protein Thy-1 was found in microdomains at steady state, as judged by a single particle tracking analysis (Sheets et al., 1997). Once bound to its receptor, proaerolysin must move about to encounter its processing enzyme furin in the phosphoglyceride region of the plasma membrane. This lateral movement is favored by the lipid anchor of the receptor. It has indeed been shown that GPI-anchored proteins have a higher lateral mobility within a phospholipid bilayer than their transmembrane counterpart (Zhang et al., 1992). Subsequent encounters between toxin subunits, required for the aerolysin oligomerization step, is favored by the capacity of the GPI-anchored receptors to associate with cholesterol-rich microdomains and to remain confined to them for several seconds (Sheets et al., 1997). This clustering device enables the toxin to concentrate locally and therefore to oligomerize efficiently. Cholesterol-rich microdomains, thus, appear to act as concentration platforms and offer sites of assembly for the aerolysin heptamer. In addition, microdomains might provide an ideal location for membrane insertion of the toxin. At the junctures between cholesterol-sphingolipid–rich domains and fluid phase phosphoglyceride domains, there are probably unfavorable energetic effects (Brown, 1998) that locally weaken the lipid bilayer and might favor membrane penetration. The route followed by aerolysin at the cell surface nicely illustrates the highly dynamic behavior of raft components and the transitory nature of the interaction of GPI-anchored proteins with cholesterol-rich microdomains. The present work also provides functional evidence for the existence of rafts at the surface of living cells.

Appendix

To estimate the factor by which proaerolysin becomes concentrated when binding to the BHK cell surface, the following calculation was made: 2.5 ml of ^{125}I-proaerolysin at a concentration of 0.4 nM was added to a 56.7-cm^2 confluent petri dish. We found routinely that ~5% of ^{125}I-proaerolysin was bound at 37°C, i.e., 10^{-13} mol of toxin. In a calculation similar to that described by McLaughlin and Aderem (1995), we considered that the bound toxin was located in a thin, but finite, region adjacent to the membrane, of thickness d, where d is of molecular dimensions ($1 < d < 10$ nm). This surface phase corresponds to the Guggenheim model of a surface. Therefore, upon translocation of 10^{-13} mol of toxin to volume of d = 10 nm × 56.7 cm^2, the concentration in the surface phase would be 1.3 μM, leading to a concentration factor of ~1,500, when compared with the bulk toxin.

We would like to thank Marc Fivaz, Pierre-Etienne Glauser, Franck Perez, Manuel Rojo, and Jean Gruenberg for critical reading of the manuscript, and Thomas Buckley for providing us with the aerolysin overproducing strain and the antiproaerolysin mAbs.

This work has been supported by grants from the Swiss National Science Foundation and from the Fondation Ernst et Lucie Schmidheiny to F.G. van der Goot.

Submitted: 9 April 1999
Revised: 17 August 1999
Accepted: 26 August 1999

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