Channel Formation by the Glycosylphosphatidylinositol-anchored Protein Binding Toxin Aerolysin Is Not Promoted by Lipid Rafts*

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Glycosylphosphatidylinositol-anchored proteins may be concentrated in membrane microdomains (lipid rafts) that are also enriched in cholesterol and sphingolipids. The glycosyl anchor of these proteins is a specific, high affinity receptor for the channel-forming protein aerolysin. We wished to determine if the presence of rafts promotes the activity of aerolysin. Treatment of T lymphocytes with methyl-β-cyclohexextrin, which destroys lipid rafts by sequestering cholesterol, had no measurable effect on the sensitivity of the cells to aerolysin; nor did similar treatment of erythrocytes decrease the rate at which they were lysed by the toxin. We also studied the rate of aerolysin-induced channel formation in liposomes containing glycosylphosphatidylinositol-anchored placental alkaline phosphatase, which we show is a receptor for aerolysin. In liposomes containing sphingolipids as well as glycerophospholipids and cholesterol, most of the enzyme was Triton X-100-insoluble, indicating that it was localized in rafts, whereas in liposomes prepared without sphingolipids, all of the enzyme was soluble. Aerolysin was no more active against liposomes containing rafts than against those that did not. We conclude that lipid rafts do not promote channel formation by aerolysin.

The possibility that lateral phase separations of specific bilayer components might lead to the occurrence of microdomains in cell membranes has received a great deal of recent attention (1–4). These “lipid rafts” are largely defined by their resistance to extraction with nonionic detergents (5–7). They are enriched in sphingolipids and cholesterol as well as in a number of membrane proteins, including several signal-transducing molecules and glycosylphosphatidylinositol (GPI)1-anchored proteins (7–11). Some members of this latter group of proteins are themselves involved in cell signaling, while some are enzymes and others have less well defined functions (12–16).

The channel-forming protein toxin aerolysin and its inactive precursor proaerolysin have the unique ability to bind specifically and with high affinity to GPI-anchored proteins on the surfaces of target cells (17–20). Once bound, proaerolysin may be converted to aerolysin by surface proteases (21). Bound aerolysin then forms heptamer oligomers that can insert into the plasma membrane, producing discrete channels (22). Because binding effectively concentrates the toxin on the cell surface, promoting oligomerization, cells that display GPI-anchored proteins are far more sensitive to aerolysin than those that do not (23, 24). Thus, normal T lymphocytes, which contain several GPI-anchored proteins that bind aerolysin, including Thy-1, are killed by 1-h exposure to 10−10 M aerolysin or proaerolysin, whereas T lymphocytes that lack GPI-anchored proteins because they are unable to synthesize the anchor are approximately 104-fold less sensitive. Similarly, we have shown that channel formation in artificial lipid bilayers occurs at far lower aerolysin concentrations if the bilayers contain incorporated GPI-anchored proteins, such as Thy-1 from brain or lymphocytes, or the erythrocyte aerolysin receptor, a novel aerolysin-binding GPI-anchored protein purified from erythrocytes (17, 18).

Recently, it has been proposed that lipid rafts promote channel formation by aerolysin because the increased density of GPI-anchored proteins therein leads to higher toxin concentrations than elsewhere on the cell surface, thereby, it was argued, increasing the rate of oligomerization (25). However, only circumstantial evidence was presented to support the proposal. It was shown that aerolysin comigrates with the Triton X-100-insoluble fraction upon density gradient centrifugation, which is consistent with the fact that GPI-anchored proteins also tend to migrate there, and with our observation that aerolysin binds these proteins with high affinity (17–20). It was also shown that treating cells with a cholesterol-lowering agent, which is known to lower the amount of GPI-anchored protein that is detergent-insoluble, also lowered the amount of detergent-insoluble aerolysin (25). Surprisingly, however, no comparison was made of the rate of channel formation by the toxin in the normal and treated cells.

Although it is possible that the concentration of GPI-anchored proteins in rafts might promote oligomerization of aerolysin, rafts could conceivably have the opposite effect. These regions are thought to be enriched in saturated lipids, so that the lateral mobility of GPI-anchored proteins may actually be lower when they are in rafts than when they are in the bulk of the membrane (2, 3). Restricted motion of bound aerolysin would tend to lower oligomerization rates. In any case, whether or not aerolysin binding to raft-associated GPI-anchored proteins does affect the kinetics of oligomerization of the toxin, it seems unlikely that there would be a significant change in the overall rate of channel formation. This is because binding rather than oligomerization is the rate-limiting step in channel formation, especially at low toxin concentrations (26), so that any influence of lipid rafts on oligomerization would probably be masked.

In the present study, we looked for direct evidence of an

Received for publication, April 3, 2000, and in revised form, April 18, 2000
Published, JBC Papers in Press, April 18, 2000, DOI 10.1074/jbc.M002785200

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Printed in U.S.A.

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‡ The abbreviations used are: GPI, glycosylphosphatidylinositol; PLAP, placental alkaline phosphatase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CH, cholesterol; SM, sphingomyelin; DMEM, Dulbecco’s modified Eagle’s high glucose medium; PBS, phosphate-buffered saline.

This paper is available online at http://www.jbc.org

Published, JBC Papers in Press, April 18, 2000, DOI 10.1074/jbc.M002785200
effect of lipid rafts on channel formation by aerolysin. Cell
sensitivity to the toxin was compared before and after treat-
ment with methyl-β-cyclohexetrin, which abolishes lipid rafts by
reducing plasma membrane cholesterol levels (27). We found
that the sensitivity of a T cell line was unaffected by cholesterol
extraction; nor was the sensitivity of erythrocytes decreased by
cholesterol removal. We also studied liposomes containing in-
corporated GPI-anchored placental alkaline phosphatase
(PLAP), which we show acts as an aerolysin receptor. Lipo-
somes containing PLAP associated with rafts were no more
sensitive to aerolysin than liposomes that were raft-free.

EXPERIMENTAL PROCEDURES

Materials—Lipid phosphatidyicholine (PC) and phosphatidyethano-
lamine (PE) and brain sphingomyelin (SM) were obtained from Avanti
Polar Lipids. Cholesterol and a crude preparation of human placental
alkaline phosphatase were purchased from Sigma. Proaerolysin and the
inactive variant Y221G were purified as described previously. The
purified variant was labeled with the fluorescent probe Alexa 488
(Molecular Probes, Inc., Eugene, OR), using a procedure provided by
the manufacturer.

Cell Culture—The murine lymphocyte cell line ELA was generously
provided by Dr. R. Hyman (Salk Institute). Cells were suspended in Dul-
becco’s modified Eagle’s high glucose medium (DMEM) supplemented
with bovine fetal calf serum (10%, v/v), streptomycin (100 µg/ml), and
carbenicillin (100 units/ml) with 5% CO2 at 37 °C.

Cholesterol Extraction with Methyl-β-cyclohexetrin—ELA cells at 2 × 106/ml were washed twice in neat DMEM and then incubated with
500 µl of 0.5% methyl-β-cyclohexetrin in DMEM for 30 min at 37 °C,
rotating end over end. Following extraction, half of the cells were
washed twice in DMEM and used in the cytotoxicity assay and for flow
cytometry; the other half were washed twice in PBS, and then a cho-
lesterol determination (Cholesterol 20; Sigma) was performed on them.

Measurement of Cholesterol—Lipid rafts were extracted by detergent
incubation of 106 cells/ml in DMEM, 0.5% bovine serum albumin) were incubated
with 10–5 M Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton
X-100 (w/v), containing a protease inhibitor mixture (1% phenylmeth-
ylethylsulfonyl fluoride, 2 µg/ml aprotinin, 2.2 µg/ml leupeptin, and 1 µg/ml
dipeptidyl carboxypeptidase II), prepared in PBS (pH 7.4). The cell
pellet was resuspended in 450 µl of 0.1% Triton X-100 by adding cold 20 mM HEPES, pH 7.4. Following warming and centrifuging to separate the phases once more, protein was precipitated from the detergent-rich phase by adding 5
mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA to final concentrations of 333 and 7.66
mM HEPES, pH 7.4. Following centrifuging and washing of the pellet,
protein was determined as described previously (28).

Flow Cytometry—Control cells and cells that had been treated with
methyl-β-cyclohexetrin for 30 min at 37 °C were exposed to 10–5 M Alexa
488-labeled Y221G proaerolysin for 30 min on ice. They were then washed twice with PBS and analyzed by flow cytometry.

PLAP Purification—Twenty-five mg of human PLAP were dissolved
in 50 ml of 1% Triton X-114 in PBS containing 1 mM phenylmethyl-
sulfonyl fluoride by incubating for 20 min on ice. The extract was separated into detergent-rich and aqueous phases by warming the sample to 37 °C
for 10 min and then centrifuging in a JA17 rotor (Beckman) for 10 min
at 10,000 rpm and 23 °C. The detergent-rich phase was cooled and
diluted back to 1% Triton X-114 by adding cold 20 mM HEPES, pH 7.4.
Following warming and centrifuging to separate the phases once more,
protein was precipitated from the detergent-rich phase by adding 5
mM EDTA, 1% Triton X-100, and centrifuging in a JA17 rotor for 10 min
at 10,000 rpm at 23 °C. The detergent-rich phase was cooled and
diluted back to 1% Triton X-114 by adding cold 20 mM HEPES, pH 7.4
was added to final concentrations of 5 nM in stirred cuvettes containing 1.5 ml of 0.8% (w/v) washed cells in PBS. The rate of hemolysis was monitored by measuring the
decrease in optical density of the erythrocyte suspensions at 600 nm and
37 °C as a function of time. Readings were made using a Varian Cary I
recording spectrophotometer as described previously (29).

The aerolysin concentration dependence of rat erythrocyte hemolysis
was determined as described previously (28).

Liposome Preparation—Lipid films containing 12 µmol of total lipid in
the proportions 5 PC:3 PE:3 CH, 3 PC:3 PE:3 CH:2 SM, 4 PC:3 PE:3
CH, and 3 PC:3 PE:3 CH:1 SM were suspended in PBS. The rate of hemolysis was monitored by measuring the decrease in optical density of the erythrocyte suspensions at 600 nm and 37 °C as a function of time. Readings were made using a Varian Cary I recording spectrophotometer as described previously (29).

The aerolysin concentration dependence of rat erythrocyte hemolysis
was determined as described previously (28).

Detection of Proteins by Sandwich Western Blotting—Samples were
analyzed by SDS-polyacrylamide gel electrophoresis (32) and blotted.
Blots were incubated with proaerolysin, followed by polyclonal anti-aero-
lysin and anti-rabbit horseradish peroxidase according to our published
procedure (18). They were then developed by enhanced chemilumines-
cence (Amersham Pharmacia Biotech).
Lipid Rafts Do Not Promote Aerolysin Action

RESULTS

Some Aerolysin Is Recovered in a Detergent-insoluble Cell Fraction—One of the few easily measured properties of lipid rafts is their insolubility in some nonionic detergents (5, 7). The raft lipids and proteins are thought to remain associated in Triton X-100, and the insoluble complexes can be separated from other cell components by flotation in sucrose density gradients (5). Since proaerolysin binds very tightly with GPI-anchored proteins, which partially associate with the insoluble complexes, some proaerolysin should be found in the floating fraction after extraction of cells that have been pretreated with the protein. Such an association was first reported using baby hamster kidney cells and presented as evidence that rafts favor channel formation by aerolysin (25). The results in Fig. 1A show that detergent extraction of EL4 cells pretreated with proaerolysin also led to a detergent-insoluble fraction, corresponding to rafts, that contained some of the protoxin. Not all of the protein was recovered with the floating fraction. Some remained at the bottom of the centrifuge tube, indicating that it was extracted with detergent and therefore not raft-associated. A similar distribution of the GPI-anchored protein Thy-1 was observed (data not shown). This is consistent with the generally held view that only a fraction of the plasma membrane’s GPI-anchored proteins are localized in rafts at any given time. It was not possible to make a direct comparison of our results with those obtained with baby hamster kidney cells, since in that study gradient fractions were normalized to constant total protein, thereby heavily weighting the amount of aerolysin apparently associated with the floating fraction in the SDS-polyacrylamide gels (25).

The fact that detergent extraction of cells that had been incubated with aerolysin resulted in the recovery of some of the toxin in a detergent-insoluble fraction is not necessarily evidence that there is a real and physiologically significant association of the toxin with rafts in situ. Thus, when aerolysin was added after the cells had been disrupted with Triton X-100, the amount recovered in the low density detergent-insoluble fraction did not differ very much from the amount recovered in this fraction when the cells had been preincubated with the toxin before extraction (Fig. 1, compare A and B).

RAFT DEPLETION BY CHOLESTEROL EXTRACTION DOES NOT AFFECT CHANNEL FORMATION IN LYMPHOCYTES—There is considerable evidence that the detergent-insoluble fraction, or lipid rafts, can be greatly reduced by lowering the cholesterol content of the membrane. This can be accomplished by treating cells with the cholesterol-sequestering agent methyl-β-cyclodextrin (27). Extraction of EL4 cells with 10 mM methyl-β-cyclodextrin for 30 min led to a 45% decline in cholesterol levels and a striking change in the amount of Thy-1 detected in the detergent-insoluble fraction (data not shown), evidence that rafts had been greatly reduced. Flow cytometric analysis of the treated and control cells using a fluorescently labeled toxin indicated that cholesterol extraction had little or no effect on aerolysin binding (Fig. 2). This was not surprising, since there is no reason to believe that there should be any difference in the binding of the protein to GPI-anchored proteins whether they are located in rafts or distributed on the rest of the membrane surface. More importantly, the results in Fig. 3 show that the depleted cells, containing considerably less cholesterol and detergent-insoluble GPI-anchored protein, were no less sensitive to the toxin than the untreated cells.

Cholesterol Depletion Does Not Decrease the Sensitivity of Erythrocytes to Aerolysin—We also studied the effect of cholesterol depletion on the sensitivity of human erythrocytes. Erythrocytes are lysed by aerolysin at concentrations similar to those that kill EL4 cells (Fig. 3), although they contain different aerolysin-binding GPI-anchored proteins. This too is not surprising, since aerolysin binds to the anchor, which has a constant core structure from cell to cell and species to species. Treatment with 3.5 mM methyl-β-cyclodextrin for 60 min removed 90% of the erythrocyte cholesterol, a higher percentage than is removed from other cell types, presumably because erythrocyte cholesterol is entirely associated with the plasma membrane. Remarkably, the nearly complete extraction of the steroid from the cells did not lower the rate at which they were lysed by aerolysin (Fig. 4), despite the fact that the amount of raft-associated GPI-anchored protein, if any, must have been very small.

RAFTS IN LIPOSOMES DO NOT PROMOTE CHANNEL FORMATION—The above results lend no support to the idea that lipid rafts have an important role to play in channel formation by aerolysin. However, although it seemed unlikely that the erythrocytes used to obtain the data in Fig. 4 could contain any significant raft population after extraction of 90% of their cholesterol, we could not exclude the possibility that a small raft population remained. In order to compare membranes totally lacking rafts with membranes that contain a detergent-soluble fraction, we decided to use large unilamellar vesicles containing a GPI-anchored protein that acts as an aerolysin receptor. We have previously shown that Thy-1 is an excellent receptor for aerolysin when it is incorporated into liposomes; however, for the present study we wished to use a protein that could be more easily quantitated, so we first determined if GPI-anchored placental alkaline phosphatase could substitute for Thy-1. The results in Fig. 5A show that the addition of 36 mM aerolysin to liposomes containing incorporated PLAP resulted in rapid dye release, whereas liposomes without PLAP were not affected by this concentration of the toxin. We obtained a very similar pattern with liposomes containing incorporated Thy-1 (18). Having shown that PLAP is an aerolysin receptor, we then incorporated the enzyme into liposomes of different lipid compositions. Most of the PLAP in the 3:3:3:2 (PC:PE:CH:SM)
vesicles was detergent-insoluble, evidence for the presence of rafts, whereas all of the enzyme in liposomes lacking SM was soluble in Triton X-100. Vesicles made with half as much SM had approximately half as much detergent-insoluble PLAP (data not shown). The ability to prepare liposomes with and without rafts allowed us to determine directly whether or not these lipid structures have any pronounced effect on aerolysin sensitivity.

The rates of channel formation by aerolysin were next compared in liposomes lacking raft-associated PLAP or containing moderate (Fig. 5A) or large amounts (Fig. 5B) of the detergent-insoluble enzyme. It may be seen that there were only minor differences in the rate of dye release regardless of the liposome composition. Release from the liposomes containing large amounts of raft PLAP was actually somewhat slower than from the corresponding liposomes lacking sphingomyelin (Fig. 5B). The two lipid populations contained very similar proportions of PLAP to lipid, although the SM-containing population was somewhat smaller. Comparable results were obtained when lower concentrations of aerolysin were used (data not shown).

DISCUSSION

Although the results in this paper cast no direct light on the existence or significance of lipid rafts in cell membranes, they clearly show that if these structures exist, they have little or no influence on the rate of channel formation by aerolysin, either in whole cells or in liposomes. The result is not surprising for two reasons. First, even if rafts did alter the rate of aerolysin oligomerization, we would not expect to see an effect on cells unless oligomerization becomes the rate-limiting step in channel formation. Normally, binding is rate-limiting, and there is no reason to believe that GPI-anchored proteins clustered in rafts would bind the toxin any better than when they are distributed on the cell surface. In fact, the results in Fig. 2 show that binding is not affected by raft disruption. Second, it is not intuitively obvious that, even if oligomerization were the rate-limiting step in channel formation, it would occur more quickly if the receptors were clustered in rafts. Membrane GPI-anchored proteins could be less mobile when they occur in these regions, because of the increased levels of relatively saturated fatty acids found there (7). The reduced mobility could lower the rate of oligomerization of the bound toxin. In any case, GPI-anchored proteins are also located in the bulk of the lipid bilayer.
bilateral, where they are apparently very mobile, some have speculated even more mobile than transmembrane proteins (3, 14). They appear to simply pause in rafts as they move about the cell surface; at any given time, only a fraction is thought to be localized in rafts (3, 33).

The results we obtained with erythrocytes extracted with methyl-β-cyclodextrin allow us to comment on the role of cholesterol in channel formation by aerolysin. There has been a recent report that aerolysin is inhibited when preincubated with cholesterol, which was taken as evidence that the toxin can interact with the steroid (34). Earlier we had shown that aerolysin is capable of forming channels in cholesterol-free liposomes, indicating that it has no absolute requirement for the steroid (35). Here we found that removing nearly all of the cholesterol from the cell did not lower the rate of channel formation. Thus, it would seem that cholesterol plays no significant role in the action of aerolysin.

In conclusion, the unusual ability of aerolysin to bind to GPI-anchored proteins may provide a new tool to study the chemistry and biology of lipid rafts, but there is no reason to believe that these structures are important in the process of channel formation by the toxin.

Acknowledgments—We thank Félix González and Alicia Alonzo for helpful discussions. The technical assistance of Ryan Barry is gratefully acknowledged.

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