Glycosylphosphatidylinositol Anchors of Membrane Glycoproteins Are Binding Determinants for the Channel-forming Toxin Aerolysin

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Cells that are sensitive to the channel-forming toxin aerolysin contain surface glycoproteins that bind the toxin with high affinity. Here we show that a common feature of aerolysin receptors is the presence of a glycosylphosphatidylinositol anchor, and we present evidence that the anchor itself is an essential part of the toxin binding determinant. The glycosylphosphatidylinositol (GPI)-anchored T-lymphocyte protein Thy-1 is an example of a protein that acts as an aerolysin receptor. This protein retained its ability to bind aerolysin when it was expressed in Chinese hamster ovary cells, but could not bind the toxin when expressed in Escherichia coli, where the GPI anchor is absent. An unrelated GPI-anchored protein, the variant surface glycoprotein of trypanosomes, was shown to bind aerolysin with similar affinity to Thy-1, and this binding ability was significantly reduced when the anchor was removed chemically. Cathepsin D, a protein with no affinity for aerolysin, was converted to an aerolysin binding form when it was expressed as a GPI-anchored hybrid in COS cells. Not all GPI-anchored proteins bind aerolysin. In some cases this may be due to differences in the structure of the anchor itself. Thus the GPI-anchored proteins procyclin of Trypanosoma congolense and gp63 of Leishmania major did not bind aerolysin, but when gp63 was expressed with a mammalian GPI anchor in Chinese hamster ovary cells, it bound the toxin.

Glycosylphosphatidylinositol (GPI)-anchored proteins are common components of the external surfaces of eucaryotic cells (1–3). The first described was the variant surface glycoprotein (VSG) from Trypanosoma brucei brucei, which protects the surface of the bloodstream form of the parasite (4). Since then a great many mammalian proteins have been shown to be anchored to the cell surface in the same way as VSG (3). Some of these proteins are enzymes, such as placent al alkaline phosphatase (5) and erythrocyte acetylcholinesterase (6), whereas others appear to be involved in cell adhesion, complement regulation, or even in transport (1). Still others are thought to play roles in communication between cells (7–11), and there is accumulating evidence that clustering of some GPI-anchored proteins may represent a new mechanism of signal transduction. Thus macrophages and neutrophils are activated by antibody cross-linking of CD14 (lipopolysaccharide receptor) and CD16 (Fc-γRIIIb), which leads to cytokine expression and oxidative burst (12–14), and cross-linking of several T-lymphocyte GPI-anchored proteins produces profound regulatory signals (15–18).

So far the structures of the GPI anchors of only a few proteins have been determined. All of the anchors contain a core of ethanolamine-HPO₄₂⁻, which has been shown to be required for the virulence of Aeromonas hydrophila (reviewed in Ref. 21). Once released, the proton is activated by proteolytic nicking. Aerolysin binds to sensitive cells and oligomerizes, inserting into the membrane and forming discrete channels that breach the permeability barrier. We have shown that sensitive cells contain receptors that bind both proaerolysin and aerolysin with high affinity (Kᵣ ≈ 10⁻⁹ M; Ref. 22). In the case of mammalian erythrocytes, the receptor is a 47-kDa glycoprotein that has N-terminal sequence homology to a group of recently characterized proteins that are involved in ADP-riboseylation reactions (23). We have also shown that the major surface glycoprotein Thy-1 is a receptor for aerolysin in mouse T-lymphocytes (24).

The erythrocyte aerolysin receptor (EAR) and Thy-1 are apparently unrelated to each other in primary structure and in function. Although both proteins are N-glycosylated, we have found that the N-linked sugars are not required for toxin binding. However, Thy-1 and EAR do share one remarkable feature, both are attached to the cell surface with GPI anchors. This is also true of an 80-kDa aerolysin-binding protein in baby hamster kidney cells.² In this paper we show that this is not a coincidence, and we provide evidence that the anchor itself is required for aerolysin binding.

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² L. Abrami, M. Fivaz, R. G. Parton, and F. G. van der Goot, submitted for publication.

The abbreviations used are: GPI, glycosylphosphatidylinositol; EAR, erythrocyte aerolysin receptor; VSG, variant surface glycoprotein; NCAM, neural cell adhesion molecule; CD, cathepsin D; CHO, Chinese hamster ovary; COS, African green monkey kidney cell line; PI-PLC, phosphatidylinositol-specific phospholipase C; PAG, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; HF, hydrofluoric acid.
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EXPERIMENTAL PROCEDURES

**Bacterial Strains, Mammalian Cell Lines, and Their Growth Conditions**—Escherichia coli strains JM109 and BL21(DE3)/pLysS (Novagen) were used as hosts for plasmid amplification and gene expression, respectively. Both strains were grown in LB medium at 37 °C and 300 rpm using a rotary shaker. Where appropriate, ampicillin, chloramphenicol, and kanamycin were added at concentrations of 100, 34, and 40 μg/ml, respectively. CHO cells were grown in Dulbecco’s modified Eagle’s high glucose medium. Both media were supplemented with bovine fetal calf serum (10%, v/v), streptomycin (100 μg/ml), and penicillin (100 units/ml). All cell lines were grown in 5% CO₂ at 37 °C.

**Plasmid Construction and Gene Expression**—The PCR primers GG-GAACATCATGGAACAGTTGTAAC and GCCGAATTCACACACAGTTGACATTTTGC were used to isolate the DNA sequence encoding the mature peptide of Thy-1 from the mouse cDNA clone pT128U.TM8.5 (Ref. 27; also a gift from Dr. R. Hyman). The primers were designed so that the cloned gene began with an added methionine codon (ATG) to initiate translation and ended with an added stop codon (TGA). The PCR product was ligated into the prokaryotic expression vector pET29a (Novagen), to obtain plasmid pM24 which was amplified in JM109, isolated, and retransformed into BL21(DE3)/pLysS. Expression of recombinant protein was performed according to the protocol provided in the Sequenase kit from U.S. Biochemical Corp.

**Expression of recombinant protein** was induced by adding the sample to frozen saturated lithium hydroxide, and the mixture was incubated for 2 h at 37 °C. The sensitivity of recombinant protein was assessed using a Western blotting procedure.

**PI-PLC Treatment**—To select CHO-K1 cells for stable expression a CaCl₂ precipitation method (31) or into COS cells for transient expression using a DEAE-dextran and chloroquine method (32). To select CHO-K1 cells, 500 μg/ml Geneticin (G418; Life Technologies, Inc.) was added to the growth medium. Transfected cells were allowed to grow for 3 days after transfection and washed once with 4.3 mM Na₃HPO₄, 1.4 mM KH₂PO₄, pH 7.4, containing 137 mM NaCl, 5.2 mM KCl. Aliquots of resuspended cell pellets were then used to detect proaerolysin-binding proteins by sandwich Western blotting as described below. The entire insert sequence in pRc-Thy-1 and pM24 was confirmed by DNA sequencing using the chain termination method and the Sequenase kit from U.S. Biochemical Corp.

**Detection of Proteins by Western Blotting**—Sandwich Western blotting was used to detect proaerolysin-binding proteins as described previously (24). Briefly, cell or protein samples were separated by SDS-PAGE and blotted onto nitrocellulose. The blots were probed with a rabbit polyclonal anti-proaerolysin antibody and anti-rabbit horseradish peroxidase. The surface protein gp63 expressed in CHO cells and in Leishmania donovani (anti-cross-reacting determinant; Oxford Glycosystems, Ref. 36). The VSG was a kind gift from Dr. Terry Pearson (University of Virginia). The glycoprotein (150 μg) was incubated with 100 μl of 50% aqueous HF at 0 °C for 2 h. A control sample was incubated with water under the same conditions. The HF was neutralized by adding the sample to frozen saturated lithium hydroxide, and the precipitate of lithium fluoride was removed by centrifugation. The pellet was washed twice with 50 μl of distilled water, and the aqueous portions were combined and desalted over a PD-10 (G-25) column equilibrated in 20 mM HEPES, pH 7.4, containing 150 mM NaCl. Proaerolysin binding was assessed following Western blotting, after SDS-PAGE of the void volume fraction. Anchor removal was confirmed using a commercial antibody that detects the presence of the GPI anchors (anti-cross-reacting determinant; Oxford Glycosystems, Ref. 36).

**RESULTS**

**T-lymphocytes Lacking Thy-1 Remain Sensitive to Aerolysin**—We have shown that Thy-1 binds aerolysin with high affinity and that liposomes containing incorporated Thy-1 are more sensitive to the toxin (24). We have also found that EL4 cell lines that cannot add GPI anchors to membrane proteins resist aerolysin’s action, and we had assumed that this was because they lacked Thy-1 (24). We were therefore surprised to discover that the mouse mutant T-lymphocyte cell line AKR1 (Thy-1 d) which does not express Thy-1 (28), was almost as sensitive to aerolysin as was the parent strain (Fig. 1). This led us to consider the possibility that endogenous GPI-anchored proteins bind to more than one GPI-anchored protein in T-lymphocytes.

The sensitivity of the AKR1 (Thy-1 d) cell line to the toxin could then be attributed to the presence of other GPI-anchored receptors, all of which would be missing from the mutant EL4 cells we studied earlier. This explanation was supported by the results of treating cells with phosphatidylinositol-specific phospholipase C. 

**N-glycosidase Treatment**—Mouse brain homogenate, prepared as described previously (24), was treated with PI-PLC and centrifuged. A 10-μl sample of the supernatant was mixed with an equal volume of N-glycosidase incubation buffer (40 mM NaH₂PO₄, 100 mM EDTA, 1% sodium dodecyl sulfate, 10% mercaptoethanol, pH 7.5), and the mixture was boiled for 2 min. After the mixture was cooled to room temperature, 3.3 μl of a protease inhibitor mixture (0.6 mM phenylmethylsulfonyl fluoride, 60 μg/ml aprotinin, 120 μM leupeptin, and 12 μM pepstatin A) was added, followed by 2.5 μl of 10% octylglycoside and 7.5 μl of peptide N-glycosidase F (Oxford Glycosystems), containing 1.5 units of the enzyme. A control incubation was also carried out in which 7.5 μl of buffer was added in place of the enzyme. After 18 h at 37 °C, sample buffer was added, and aliquots were separated by SDS-PAGE and sandwich Western-blotting.

**Pronase Treatment of Thy-1 Incorporated into Liposomes**—Pronase treatment with 100 μg/ml Geneticin (G418; Life Technologies, Inc.) was added to the growth medium. Transfected cells were collected 3 days after transfection and washed once with 4.3 mM Na₃HPO₄, 1.4 mM KH₂PO₄, pH 7.4, containing 137 mM NaCl, 5.2 mM KCl. Aliquots of resuspended cell pellets were then used to detect proaerolysin-binding proteins by sandwich Western blotting as described below. The entire insert sequence in pRc-Thy-1 and pM24 was confirmed by DNA sequencing using the chain termination method and the Sequenase kit from U.S. Biochemical Corp.
phospholipase C, which selectively removes GPI-anchored proteins from their surfaces. It may be seen in Fig. 1 that both AKR1 cell lines became less sensitive to aerolysin after treatment with the enzyme.

Proaerolysin Also Binds to More than One GPI-anchored Protein in Mouse Brain—Sandwich Western blotting of mouse brain homogenate revealed that in addition to Thy-1, there is a 110-kDa membrane-associated protein that binds proaerolysin (lane 3, Fig. 2). Like Thy-1, this protein was solubilized by treating the homogenate with PI-PLC, indicating that it too is GPI-anchored (lanes 1 and 6). A literature search for known GPI-anchored proteins of comparable size suggested that the brain protein might be either neural cell adhesion molecule (NCAM; Ref. 40) or contactin (41). There was only a small decrease in the size of this protein when it was treated with N-glycosidase (lane 2, Fig. 2). Two bands corresponding to much smaller proteins are also visible in lane 2 after N-glycosidase treatment. The lower corresponds to completely de-N-glycosylated Thy-1, and the upper may represent partially deglycosylated Thy-1 as we have discussed previously (24), or it may represent another GPI-anchored protein. The 110-kDa aerolysin-binding protein was unambiguously identified as contactin by determining its N-terminal sequence after purifying the protein, which had been liberated from the membrane by treatment with PI-PLC.

GPI Anchoring Is a General Property of Aerolysin Receptors—The observation that T-cells contain at least one GPI-anchored protein in addition to Thy-1 that binds proaerolysin, and that the erythrocyte aerolysin receptor (23) and contactin are also GPI-anchored proteins, pointed to the remarkable possibility that the anchors themselves may be involved in proaerolysin binding. The VSG of *T. brucei brucei* was the first GPI-anchored protein to be characterized (35). Aside from its anchor, it seems to be unrelated to either Thy-1 or contactin, and it is unlikely to have any sequence homology with EAR, which appears to be a member of a small family of ADP-ribosyltransferases (23). However, VSG has a similar, though not identical GPI anchor to Thy-1, so we felt it possible that it too could bind proaerolysin if the anchor is a binding determinant. Using samples of purified VSG and an ELISA-based assay, we compared proaerolysin binding to VSG with binding to purified Thy-1. The results in Fig. 3 show that the toxin also bound the trypanosomal protein. We were easily able to detect VSG by sandwich Western blotting (not shown) in amounts comparable with the amounts of Thy-1 we have detected previously (24).

Proaerolysin Cannot Bind to Thy-1 and VSG Lacking Their GPI Anchors—To obtain more direct evidence that the GPI anchor itself was involved in proaerolysin binding, we took two approaches. In the first, we compared protoxin binding to Thy-1 expressed in *E. coli*, which is not capable of adding GPI anchors, with binding to Thy-1 expressed in CHO cells, where we would expect processing to be normal (28). The results in Fig. 4 show that proaerolysin could easily detect Thy-1 expressed in the eucaryotic cell, whereas there was no evidence of specific binding to Thy-1 expressed in the bacteria, even though, as was clear from a comparison of Coomassie-stained samples, far more Thy-1 was present in the *E. coli* samples we used. It is worth emphasizing that the far Western blotting procedure we used can detect less than 1 ng of native Thy-1 (24). Of course as well as lacking the anchor, the Thy-1 expressed in the bacteria would lack the N-linked carbohydrate that is normally present in the eucaryote. However this could not account for the difference in binding we observed, since as noted above, we have shown that the N-linked sugars apparently are not required for proaerolysin binding (24).

In the second approach, we took advantage of the fact that

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HF can selectively remove nearly the entire GPI anchor from proteins without destroying the polypeptide chain (35). The results in Fig. 5 show that treatment of VSG with HF led to a very large reduction in proaerolysin binding measured using sandwich Western blotting or the ELISA assay. The results also show that binding of a commercial antibody directed against the GPI determinant, which we used as a positive control of anchor removal, was also reduced.

Adding a GPI Anchor to a Soluble Protein Confers Proaerolysin Binding Ability—A number of groups have successfully produced hybrid proteins by fusing the DNA encoding the anchor signal for a GPI-anchored protein such as decay-accelerating factor or Thy-1 to the DNA encoding a protein that is normally not anchored in this way (27, 42, 43). Many of these hybrid proteins appear to behave like normal GPI-anchored proteins without destroying the polypeptide chain (35). The availability of the surface protease gp63 of *L. major* in its native form with its natural anchor and expressed in CHO cells (33), where it presumably has an anchor specific for the cell line, gave us the opportunity to determine the effect of different anchors on proaerolysin binding. Native gp63 has a GPI anchor similar to that of procyclin (2), and consistent with this, like the trypanosomal protein, it does not bind proaerolysin (Fig. 7). However, gp63 expressed in the CHO cell line was easily detected with proaerolysin by sandwich Western blotting, evidence that replacing the *Leishmania* anchor with a mammalian one had conferred proaerolysin binding ability on the protein.

The Anchor Alone Is Not Sufficient for Proaerolysin Binding—The proteins Thy-1, contactin, VSG, hybrid cathepsin D, and gp63 have no obvious common sequence similarities, nor is it likely that any of them are related to EAR (29). It was therefore tempting to conclude that a GPI anchor alone is sufficient for proaerolysin binding, since this is the only obvious thing that all these proteins appear to have in common. However, this would not explain why our far Western blots did not detect other erythrocyte GPI-anchored proteins such as decay-accelerating factor and acetylcholinesterase, which presumably have the same anchor as EAR (45, 46). Nor would it explain why Thy-1 is the only obvious GPI-anchored protein that binds proaerolysin in blots of AKR1 cells, despite the fact that the results presented in Fig. 1 show that these cells must
contain at least one other GPI-anchored protein that binds the toxin. These inconsistencies suggest that some GPI-anchored proteins can’t be detected by proaerolysin after SDS-PAGE, at least when present in the amounts we have used, although in their native states on the cell surface, perhaps because only very small amounts are required, they may serve as receptors. This implies that the structure of the polypeptide chain may also influence proaerolysin binding. The observation of Howard and Buckley (22) that proaerolysin binding to rat erythrocytes is reduced by treatment of the cells with proteases and the more recent observation of Cowell et al. (23) that treatment of lipid bilayers containing the rat erythrocyte receptor with proteases reduces channel formation are other reasons to believe that the protein portion of the receptor is also involved in binding.

Our ability to incorporate Thy-1 into liposomes (24) gave us the opportunity to directly assess the effect of proteolysis on proaerolysin binding, by comparing aerolysin-induced dye release from liposomes containing incorporated Thy-1 with release from the same liposomes pretreated with proteases. The results are shown in Fig. 8. It may be seen that liposomes treated with protease were resistant to aerolysin at levels that caused rapid release from control liposomes.

**DISCUSSION**

The conclusion that the GPI anchor of some membrane proteins is a binding determinant for aerolysin is remarkable, but perhaps not surprising, as it is easy to argue that GPI-anchored proteins are perfect targets for toxins like aerolysin that must bind to a cell surface and oligomerize to form channels. They are found on the exterior, apical surface of the plasma membranes of most, if not all, eucaryotic cells, and they may have unusually high lateral mobility (47). As we have pointed out previously (24), they can provide the means by which aerolysin can be concentrated on the cell surface and then move laterally to oligomerize. These are essential steps in channel formation, as the toxin is not insertion competent until oligomerization has occurred. It is noteworthy that a GPI-anchored aminopeptidase in the insect gut has been identified as the receptor for another channel-forming protein, the toxin CryIA(c) of *Bacillus thuringiensis* (48), and it will be interesting to discover whether or not the anchor is also a determinant for binding of this toxin.

It seems clear from our data that proaerolysin does not bind to all GPI-anchored proteins under all conditions. One reason for this is that the anchor’s structure plays a critical role in the interaction with the toxin, and the composition of the anchor can vary from species to species and from cell to cell. This is best illustrated by the results we obtained with gp63 (Fig. 7). The native *Leishmania* protein was unable to bind proaerolysin, whereas the protein expressed in CHO cells bound the protoxin very well, presumably because it then contained a mammalian anchor that the protoxin could recognize.

It appears that some property of the protein component of the receptor is also important in proaerolysin binding. One reason to believe this is that some proteins that should have suitable anchors do not bind the protoxin, at least after SDS-PAGE. Another is that binding is destroyed by protease treatment. Since several of the proteins that we know do bind have no apparent sequence similarities with each other, it is reasonable to conclude that proaerolysin does not recognize a simple linear sequence of amino acids. The ability to create hybrid proteins containing GPI anchors that are correctly located in the plasma membrane provides us with one approach to determining the nature of the binding determinant in the polypeptide portion of the molecule.

The fact that proaerolysin appears to recognize both specific regions of the glycosyl anchor and some property of the polypeptide chain might indicate that more than one region in the protoxin is involved in binding. The crystal structure of the protein indicates that proaerolysin contains two distinct lobes (25). Recently we have reported that the smaller lobe of proaerolysin contains a fold that is similar to a fold in the C-type lectins, suggesting that it may participate in carbohydrate binding, and we presented evidence that changing one of the amino acids in the fold could reduce binding to VSG (37). More recently we have found that a region in the large lobe of the protein also affects binding.4 These results point to the possibility that one lobe of the protein recognizes the anchor portion of the receptor and the other lobe recognizes the polypeptide.

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