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Analysis of Transmembrane Dynamics of Cholera Toxin Using Photoreactive Probes

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Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography, we have shown that ^{125}I-labeled cholera toxin binds to Newcastle disease virus. Pretreatment of Newcastle disease virus with "cold" cholera toxin (at 37°C for 30 minutes) inhibits the binding of ^{125}I-labeled toxin in a subsequent incubation (at 37°C for 30 minutes). These results suggest that cholera toxin binds to Newcastle disease virus in a specific manner. The precise receptor for toxin is unknown in Newcastle disease virus but it is presumed to be the ganglioside GM_1. We have previously shown that the photoreactive probe 12-(4-azido-2-nitrophenoxy)stearoylgucosamine[1-^{14}C] labels the membrane proteins of Newcastle disease virus. Since the reactive group of the probe, ie, N_3, resides within the membrane bilayer, studies were initiated to determine which, if any, of the subunits of cholera toxin cross the membrane of Newcastle disease virus and become radioactively labeled upon photoactivation of the probe at 360 nm. After a 15-minute incubation of cholera toxin with Newcastle disease virus containing the photoreactive probe, irradiation effected the ^{14}C-labeling of the active A_1 subunit of cholera toxin. Irradiation of cholera toxin in solution with an equivalent amount of probe but without virus resulted in no labeling of toxin subunits.

Key words: transmembrane signaling, cholera toxin, membranes, photoreactive probes, Newcastle disease virus

It is at the level of the membrane bilayer that transmembrane processes become most intractable to investigation. Most of the information on transmembrane signaling events is gathered at the cell surface, eg, receptor identification and isolation, or inside the cell, eg, cAMP production. The mechanism by which the transmembrane signal is relayed or coupled to the cytoplasmic machinery of the cell is for the most part a black box.

The purpose of this communication is to describe a protocol to elucidate the dynamics of molecules which insert into or cross the membrane bilayer. The protocol involves the

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use of photoreactive lipid analogs [1] which data from spin-label counterparts suggest are restricted to the surface monolayer of the membrane [2]. The particular probe employed in the study described here is 12-(4-azido-2-nitrophenoxy)stearoylglucosamine [1-^{14}C] (Fig. 1), λ_{max} = 245 and 360 nm. It is related to photoreactive molecules described by Khorana [3, 4], Stoffel [5, 6] and their coworkers. A reactive nitrene is generated upon the absorption of light energy ($RN_3 \rightarrow R\ddot{N}$). The nitrene is resident within the membrane bilayer, approximately 13 Å from surface carboxyl groups.

The membrane system in these studies is the membrane envelope of Newcastle disease virus (NDV), an animal virus (Fig. 2). The envelope of NDV is relatively well defined with respect to protein location, and the successful attachment of the photoreactive probe to viral membrane proteins has been demonstrated [1, 7]. It is the objective of this report to assess the ability of the photoreactive probe to attach covalently to cholera toxin components after toxin binds to the surface of NDV. We chose cholera toxin for preliminary studies since the requisite receptor, ganglioside GM_1, is known to occur in avian membranes and would presumably occur in egg-grown NDV since the viral envelope is representative of host plasma membrane in lipid and glycolipid composition [8]. Morever, NDV possesses neuraminidase activity [9] and consequently might be especially rich in GM_1 [10].

The obvious potential of the technique we describe is that it should enable us to follow the movement of proteins across specified zones within the membrane bilayer where the azido groups reside. We chose cholera toxin for these studies because it is a relatively simple toxin. Antitoxin as well as receptor is readily available, and the basic system lends itself to a variety of complementary approaches with both model and biological membrane preparations [11, 12].

MATERIALS AND METHODS

Newcastle Disease Virus

Newcastle virus (NDV) strain HP16 was propagated in ova and isolated as previously described [2].

Cholera Toxin

Cholera toxin ($A_1A_2$5B) and cholera toxoid ($A_2$5B) were purified by the method of Mekalanos, Romig, and Collier [13]. The cholera toxoid lacks the A_1 subunit of cholera

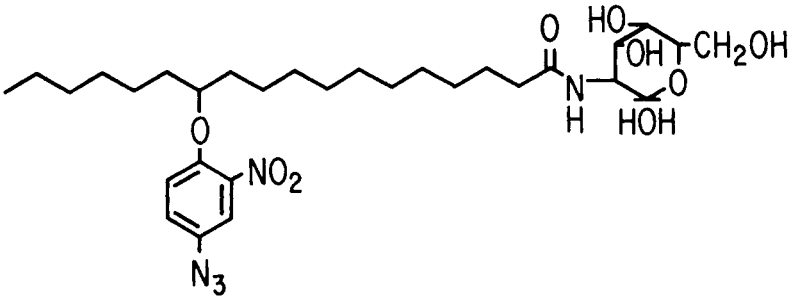

Fig. 1. Photoreactive probe: 12-(4-azido-2-nitrophenoxy)stearoylglucosamine[1-^{14}C].

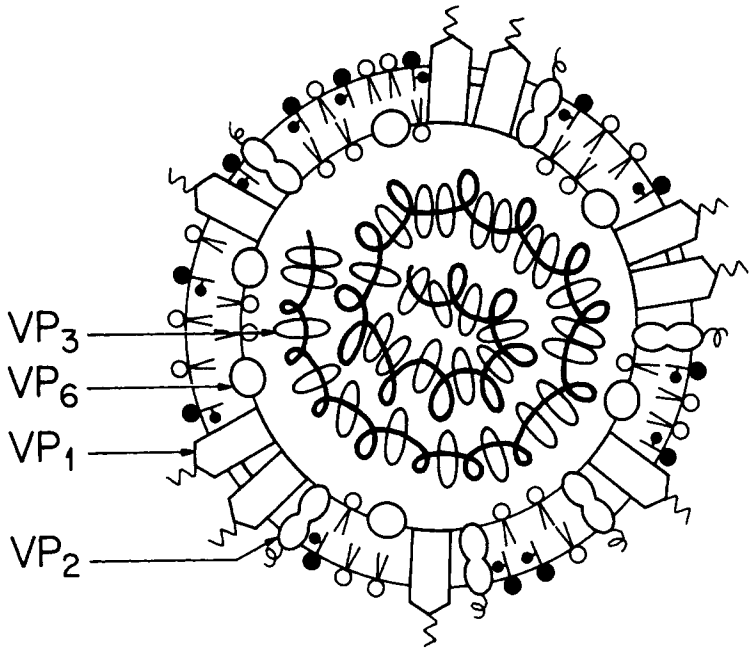

Fig. 2. Photoreactive probe inserted in the outer monolayer of Newcastle disease virus. Probable location of four major proteins is indicated.

toxin and is therefore labeled with ^{125}I more efficiently in the A_2 and B subunits than is cholera toxin. This protein, however, retains activity in binding to gangliosides [13]. Iodination of $A_2$5B was mediated by Iodogen ([14], Pierce Chemicals) in the presence of carrier-free ^{125}INa (New England Nuclear, Boston). The toxoid had a specific activity of 2 μCi/μg protein. Iodination of cholera toxin has been reported by Gill [15] to label primarily the A_1 subunit and we have confirmed this. We employed the $A_2$5B "toxoid" in these experiments because we wanted high specific activity in the binding portion of the toxin; this could only be accomplished in the absence of A_1.

Mekalanos, Romig, and Collier [13] have shown that separation of A_1 subunit from cholera toxin can give rise to a protein composed primarily of A_2 and 5B subunits (ie, $A_2$5B) which we call cholera toxoid. This protein (isoelectric point = 7.6) is apparently present as a contaminating protein in some choleragenoid (5B) preparations (pI = 7.8).

Photoreactive Probe

The synthesis, purification, and characterization of 12-(4-azido-2-nitrophenoxy)-stearoylglucosamine [1-^{14}C] were performed as previously described [1, 7]. An alternate method of coupling glucosamine to the arylazidostearic acid molecule involved the use of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ; Pfaltz and Bauer, Inc., N.Y.) in 2.5 molar excess. Glucosamine[1-^{14}C], 50 mCi/mmole, was used without carrier.

Binding Studies

One sample of NDV (50 μg as protein) was incubated alone and another with 30 μg of cholera toxin for 15 minutes at 37°C. ^{125}I-Labeled cholera toxoid ($A_2$5B) plus

^{125}I-labeled bovine serum albumin were added to both viral samples and they were incubated for 15 minutes at 37°C. The virus particles were pelleted (40,000 rpm for 30 minutes in a Beckman SW50. 1 rotor with 0.7-ml adaptors), washed and SDS-solubilized (45 minutes at 37°C) in the presence of β-mercaptoethanol. Reduced, solubilized samples were applied to discontinuous gradient gels composed of 15% acrylamide on the bottom (5 cm), 10% acrylamide on the top (5 cm), and a 2.5% stacking gel, and run using the buffer system of Laemmli [16]. After electrophoresis, the gels were stained with Coomassie blue, dried, and overlaid with x-ray film to monitor the presence of ^{125}I-labeled proteins.

Photoactivation Protocol

These experiments were carried out under red safety lights until the completion of sample electrophoresis. NDV, 160 μg as protein, was suspended in 2.4 ml of 5mM sodium phosphate-buffered saline, 1mM in EDTA, pH 7.4 (PBSE). This sample received 20 μl of an ethanolic solution of the photoreactive probe 12-(4-azido-2-nitrophenoxy)-stearoyl-glucosamine [1-^{14}C] (12.5 μCi/ml of ethanol).

The NDV sample and probe were incubated for 15 minutes at 37°C. After 15 minutes, one 1.2 ml aliquot was mixed with cholera toxin (72 μg) for 15 minutes at 37°C and another 1.2 ml aliquot was brought to 0°C and then mixed with cholera toxin (72 μg) for 15 minutes at 0°C. One 0.6 ml aliquot was removed from each 1.2 ml sample, placed in 6 × 50 mm Kimble disposable borosilicate glass culture tubes, and irradiated for one minute at 360 nm on a mineral lamp (UV-Products, San Gabriel, California). The remainder of each sample served as an unirradiated control. Cholera toxin, 36 μg/0.6 ml of PBSE (without virus), was incubated with 5 μl of probe for 15 minutes at 37°C and irradiated. The last sample received 60 μl of an ice cold 50% trichloroacetic acid solution. All samples were spun on a Beckman SW50.1 rotor (with 0.7 ml adaptors) at 149,000 g for 30 minutes. The pellets were solubilized in SDS buffer which contained β-mercaptoethanol. Solubilized samples were applied to an SDS-polyacryamide gel (10-15% acrylamide gradient with a 2.5% stacking gel). After electrophoresis, the gel was stained with Coomassie blue, photographed, and prepared for fluorography [17]. Preflashed film [18] was exposed to the gel for 30 days prior to development.

RESULTS AND DISCUSSION

To test for cholera toxin binding to NDV, we incubated one aliquot of NDV with cold cholera toxin and another aliquot without cholera toxin for 15 minutes at 37°C. ^{125}I-Labeled cholera toxoid plus ^{125}I-labeled albumin were added to both samples and they were incubated again for 15 minutes at 37°C. The viral particles were then pelleted, SDS-solubilized in the presence of β-mercaptoethanol, and electrophoresed on polyacrylamide gels (10–15% PAGE). The stained gels of virus samples incubated with only ^{125}I-labeled cholera toxoid contained cholera toxin components B (revealed by staining and autoradiography, Fig. 3) and A_2 (revealed by autoradiography; A_2 does not stain well with Coomassie blue dye). Stained gels of virus preincubated with cold cholera toxin before the addition of radioactive cholera toxoid contained cholera toxin components B and A_1, but autoradiography of the gel did not detect any ^{125}I-labeled B or A_2 subunits. The fact that pretreatment of NDV with cold cholera toxin precluded subsequent binding of ^{125}I-labeled cholera toxoid is suggestive of a specific saturable binding process. The absence of ^{125}I-albumin in the gels is further evidence for specificity of cholera toxin binding to NDV.

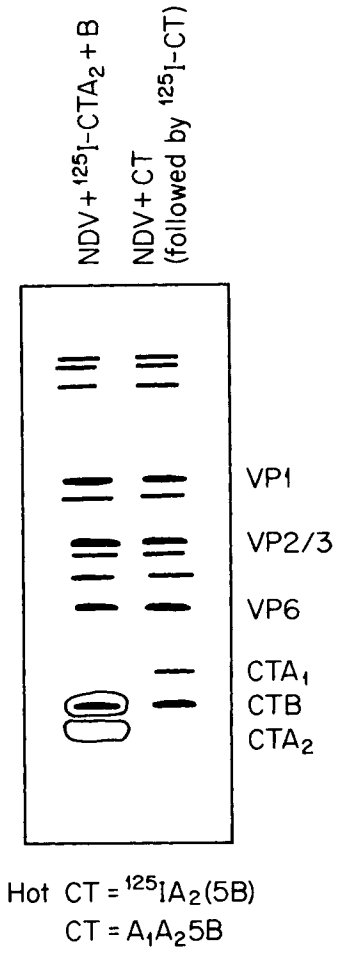

Fig. 3. SDS-polyacrylamide gel pattern of Newcastle disease virus-cholera toxin pellets. In A, virus was incubated with radioactive cholera toxoid; in B, virus was preincubated with "cold" cholera toxin before the addition of radioactive cholera toxoid. The unshaded bands represent proteins detected by autoradiography. Solid bands represent proteins stained by Coomassie Blue. (Samples were reduced with β-mercaptoethanol.)

Having demonstrated the binding of cholera toxin to NDV, we initiated an investigation into the transmembrane dynamics of the associated toxin. NDV was preincubated with ^{14}C-photoreactive probe to allow for probe insertion into the membrane envelope. After 15 minutes at 37°C, cholera toxin was added to the viral suspension and the sample allowed to incubate for 15 minutes at either 0°C or 37°C. Half of each sample was then irradiated at 360 nm for one minute; the other half of each sample served as a dark control. The viral particles were pelleted, SDS-solubilized and reduced, and electrophoresed on a continous 10-15% polyacrylamide gel as described in Materials and Methods. The results, shown in Figure 4, indicate that the radioactive label associated with cholera toxin was attached to the active A_1 subunit. Morever, the only condition which led to labeling of the A_1 subunit was a 37°C incubation with NDV for 15 minutes before irradiation. No labeling of toxin

occurred after a 0°C incubation with NDV, a condition compatible with binding but not with toxin activity. Dark controls contained no labeled proteins.

When the cholera toxin experiment was repeated without NDV, ie, with a solution of cholera toxin and ^{14}C-probe in concentrations identical to those used in the other experiments described, no detectable radioactivity was associated with toxin proteins in irradiated samples (Fig. 4, lane 1). This result provides further evidence that the photoreactive probe employed in these experiments effects very few contacts with soluble proteins. Since the cholera toxin used in these experiments contained an appreciable amount of A_1-A_2 in the form of a linear uncleaved peptide, all gel samples contain some intact A_1-A_2 (around 30,000 molecular weight) even after reduction with β-mercaptoethanol. In fact, a careful examination of Figure 4 reveals that this 30,000 MW component is also labeled under conditions which lead to labeling of the active A_1 subunit (Lane 5).

The gel system employed in these studies is not optimal for separating the proteins of NDV. The amount of radioactive probe associated with each component of NDV after irradiation has been described [7]. The majority of the radioactivity was associated with the envelope proteins and lipids.

Our results strongly suggest that the cholera toxin subunit designated A_1 extends into or crosses the membrane bilayer after the B pentamer binds to receptor gangliosides. Studies designed to distinguish these two alternatives are in progress.

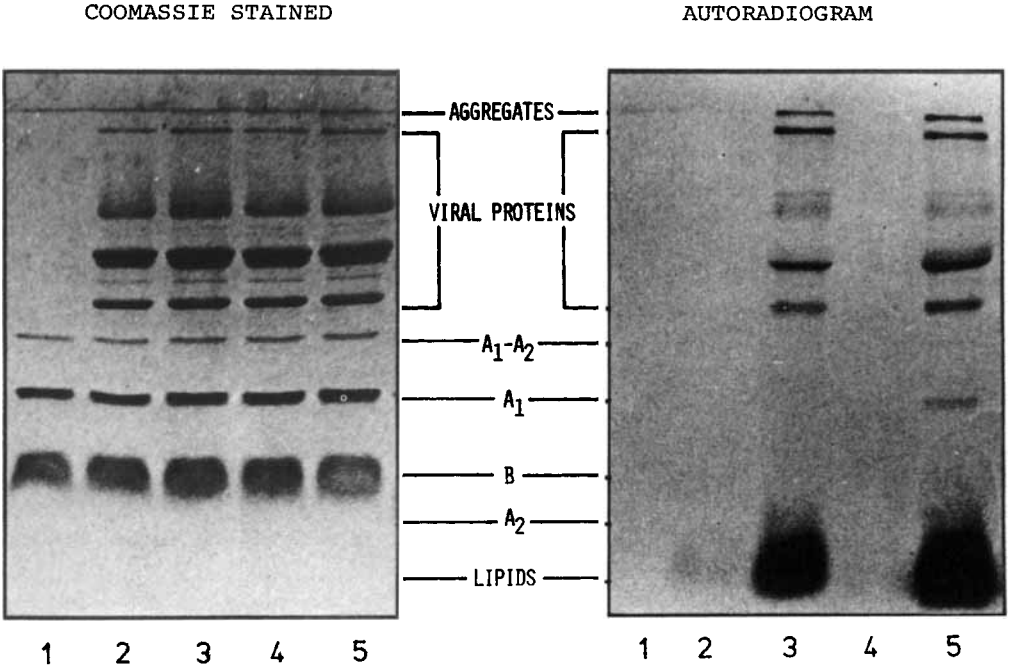

Fig. 4. Electrophoretic separation of NDV and cholera toxin samples which were incubated with the photoreactive glycolipid probe. The autoradiogram of the stained gel is shown on the right. Lane 1 contains an irradiated cholera toxin sample. Lanes 2 and 3 contain control and irradiated samples of NDV and cholera toxin which were incubated together at 0°C. Lanes 4 and 5 contain control and irradiated samples of NDV and cholera toxin which were incubated together at 37°C.

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