Carbohydrate-mediated Regulation of Interaction of Vibrio cholerae Hemolysin with Erythrocyte and Phospholipid Vesicle*

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Vibrio cholerae hemolysin is an extracellular pore-forming monomeric protein with a native molecular weight of about 60,000. In this study, we showed that the hemolysin interacted with immobilized phospholipids and cholesterol and formed oligomers in vesicles constituted from phospholipids alone with a stoichiometry identical to those produced in rabbit erythrocyte membrane. However, the hemolysin bound to glycoproteins with terminal β1-galactosyl residues and an association constant of 9.4 × 10⁷ M⁻¹ was estimated for the hemolysin-asialofetuin complex by solid phase binding assay. Oligomerization of the hemolysin in lipid bilayer converted the sugar-binding monomer to a lectin with strong carbohydrate-dependent hemagglutinating activity accompanied by inactivation of hemolytic activity and loss in ability to interact with phospholipids. There was no evidence for erythrocyte surface carbohydrates playing an essential role in interaction of the hemolysin with the cell. However, specific glycoproteins inhibited hemolysis of rabbit erythrocytes as well as interaction of the hemolysin with phospholipid. The results suggest (i) V. cholerae hemolysin is a monomer with distinct domains associated with specific binding to carbohydrates and interaction with lipids, (ii) the pore-forming property depends solely on the protein-lipid interaction with no evidence for involvement of sugars, and (iii) specific sugars can down-regulate the ability of the hemolysin to form pores in lipid bilayers.

Vibrio cholerae is a Gram-negative bacterium that produces cholera, a severe diarrheal disease of humans (1). In addition to cholera toxin (1), the critical enterotoxigenic factor of V. cholerae, the organisms generally express a protein that induces lysis of erythrocytes and other mammalian cells (2). The extruded form of this protein, the hemolysin, was purified and characterized as a monomer with a native molecular weight of 60,000 (3), cloned (4), and sequenced (5). The purified protein causes accumulation of fluid in a ligated rabbit ileal loop (6), suggesting that the protein is potentially capable of contributing to the enterotoxigenicity of V. cholerae (7).

Experiments on the mode of action of V. cholerae hemolysin on the erythrocyte membrane indicated that the protein forms transmembrane anion-selective oligomeric channels with a diameter of 0.9–1.0 nm, leading to colloid osmotic swelling of the cell and lysis (8, 9). A similar mechanism involving insertion of the monomeric cytolytic in the membrane lipid bilayer and lateral oligomerization by diffusion in the bilayer plane to a channel-like assembly with a hydrophobic outer and hydrophilic inner surface was earlier proposed for pore-forming cytolyins in general (10) and demonstrated for a wide range of cytolytic proteins such as Staphylococcus aureus α-toxin (11). Since events leading to membrane permeabilization are reproduced in artificial lipid bilayers constituted from phospholipids alone (8, 12), nonlipid constituents of the membrane such as proteins and carbohydrates do not appear to be critically involved in the process. However, there is evidence which indicates that glycoconjugates do affect pore-forming activity of cytolyins. For example, S. aureus α-toxin is inhibited by GM₁-ganglioside (13) and band 3 anion transport glycoprotein of the erythrocyte membrane (14), and Aeromonas hydrophila hemolysin is inactivated by erythrocyte membrane glycopeptides (15). These observations suggest that specific carbohydrates might regulate the interaction of the cytolyins with membrane bilayers and their oligomerization behavior. However, the possible existence of a carbohydrate-dependent regulatory mechanism for a primarily lipid-mediated process has not been studied or even recognized.

In this study, we have showed that V. cholerae hemolysin is a lectin-like protein with a preference for the terminal β1-galactosyl moiety of glycoproteins. Our results provided evidence that specific glycoconjugates can regulate the self-assembly of the hemolysin in the erythrocyte membrane and artificial lipid bilayers.

EXPERIMENTAL PROCEDURES

Materials—V. cholerae strain V₂ used for the isolation of the hemolysin was a clinical non-01 isolate, a generous gift from R. Sakazaki, Tokyo, Japan. Brain heart infusion (dehydrated medium) was from Difco. Glycoproteins, enzymes, lectins, and sugars were from Sigma. Chromatographic matrices, gel electrophoresis chemicals, and standard protein molecular weight markers were from Pharmacia, Uppsala, Sweden.

Protein Purification—V. cholerae non-01 V₂ was cultured in brain heart infusion broth at 37°C with shaking for 18 h. Bacteria were removed by centrifugation at 20,000 × g for 15 min at 4°C, and the culture supernatant was concentrated 50-fold by filtration through a PM-10 membrane (Amicon). Lipid-protein vesicles in the ultrafiltrate were removed by sedimentation at 100,000 × g for 2 h. The supernatant was fractionated by 50% saturation with (NH₄)₂SO₄, and the crude hemolysin was subjected to hydrophobic interaction chromatography on phenyl-Sepharose CL-4B column (38 × 1.6 cm) equilibrated with 25 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.6 (buffer A). The column was washed with 3 bed volumes of buffer A, and the adsorbed proteins were eluted with a linear 0–60% ethylene glycol gradient. Fractions with hemolytic activity eluted as a sharp peak at 30% ethylene glycol concentration and were pooled, concentrated by ultrafiltration, and applied on a Bio-Gel P-100 column (39 × 1.6 cm) equilibrated with 100 mM NaCl in buffer A. The elution volume of the hemolysin corresponded to a molecular weight of 60,000 as determined by calibrating the column with a mixture of bovine serum albumin, ovalbumin, chymotrypsinogen, and lysozyme. Column eluates were monitored for protein concentration by absorbance at 280 nm and assayed for hemolytic activity.

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Homogeneity of the preparation was examined by polyacrylamide gel electrophoresis (PAGE)1 under native and denaturing conditions.

The oligomeric form of the hemolysin was isolated by incubating the purified hemolysin monomer (1 mg) with 5 ml of a suspension of phosphatidylcholine (PC)-cholesterol (1:1 by weight) vesicles showing a turbidity of 2.0 at 600 nm for 2 h at 25°C. The hemolysin that was not incorporated into lipid vesicles and remained free in aqueous solution was removed by centrifugation at 10,000 × g for 10 min. The hemolysin oligomer entrapped in liposome was dispersed in 1% sodium deoxycholate in buffer A and subjected to size exclusion chromatography on Sepharose S-300 (39 × 1.6 cm). The hemolysin oligomer eluted in the void volume and was dialyzed against phosphate-buffered saline (PBS, pH 7.2) for removal of the detergent.

Assay of Hemolytic and Hemagglutinating Activity—The hemolytic activity was assayed by monitoring spectrophotometrically the release of hemoglobin at 541 nm or the decrease in turbidity of the erythrocyte suspension at 650 nm (16). The hemaggulminating activity was monitored visually.

Enzyme Treatment of Erythrocytes—Rabbit erythrocytes were desialylated by incubating the cell suspension (10% v/v) in 20 mM sodium phosphate, 45 mM sodium acetate, 120 mM NaCl buffer, pH 6.0, with Clostridium perfringens neuraminidase (Sigma; 1 unit/ml) for 60 min at 37°C. Desialylated erythrocytes were washed and incubated in the same buffer with galactose oxidase (Sigma; 5 units/ml) for 2 h at 37°C (15). Bound galactose and subsequent degradation of cell surface galactose residues were confirmed by expected changes in agglutinability of cells with peanut agglutinin (18).

Preparation of Liposomes—A mixture (1:1 by weight) of PC and cholesterol was evaporated to dryness under reduced pressure, dispersed in PBS, and sonicated for 10 min at 23 kHz (Ultrasound Cell Disruptor, Microson, Secofrd). Multilamellar vesicles were centrifuged off at 12,000 × g for 10 min at 4°C.

Analytical Procedures—Protein was estimated by a modified Folin-Ciocalteu method (19) using bovine serum albumin as a standard. SDS-PAGE was performed (20) in a 12.5% or 4–20% linear polyacrylamide gradient gel. Desialylation of glycoproteins was performed by incubation in 50 mM sodium acetate, 10 mM CaCl2 buffer, pH 5.5, with C. perfringens neuraminidase (Sigma; 1 unit/ml) for 1 h at 37°C and monitored by estimation of sialic acid by the thiobarbituric acid method (21). Periodate oxidation of asialofetuin was carried out by incubating the glycoprotein (10 mg) in 25 mM sodium acetate buffer, pH 4.5, with 25 mM sodium metaperiodate at 4°C for 22 h in the dark followed by decomposition of the excess periodate by ethylene glycol (22).

Anti-hemolysin Antiserum—Antiserum to the purified hemolysin was raised by injecting male albino rabbits intramuscularly with 100 μg of the protein emulsified with Freund’s complete adjuvant (Difco) followed by three booster injections of 100 μg of protein alone at an interval of 10 days. Animals were bled 4 days after the last injection.

Solid-phase Binding Assay—Binding of the purified hemolysin to immobilized asialofetuin and lipids was quantitated by enzyme-linked immunosorbent assay (ELISA) described previously (23). Wells of polystyrene microtiter plates (Corning) were coated in triplicate with glycoprotein (1 mg/100 μl of PBS) or lipids (1 mg/100 μl of methanol) by overnight incubation at 4°C. They were washed thrice with PBS, 0.05% Tween 20, and free sites were blocked with 200 μl of defatted milk powder (5% in PBS), washed, and incubated with various concentrations of the hemolysin for 2 h at 25°C. Wells were decanted and washed as above. The hemolysin bound by the immobilized glycoprotein or lipids was estimated by sequential incubation with rabbit anti-hemolysin antisera and horseradish peroxidase-conjugated goat anti-rabbit IgG. Non-specific interaction was minimized by including in the incubation mixture defatted milk powder (1%). Color was developed with o-phenylenediamine and monitored at 492 nm. Wells coated with defatted milk powder served as blanks.

RESULTS

Hemolysin Monomer, Hemolytic Activity, and Oligomerization in Lipid Bilayer—Purified V. cholerae hemolysin induced 50% hemolysis of a 1% suspension of rabbit erythrocytes at a concentration of 33 ng/ml. As expected for a process dependent on self-aggregation of monomers (10), the equilibrium value of the percent hemolysis showed sigmoidal dependence on the hemolysin concentration and was sensitive to the amount of protein within a rather narrow concentration range from 30 to 40 ng/ml (Fig. 1A). The time course of hemolysis of rabbit erythrocytes was relatively more sensitive to the concentration of the hemolysin, with a lag phase that increased sharply with decreasing hemolysin concentration (Fig. 1B). V. cholerae hemolysin showed erythrocyte species preference, although not very pronounced, with rabbit erythrocytes being 6- and 8-fold more sensitive than human and sheep cells, respectively.

As reported by earlier workers (9), the hemolysin inserted itself in the rabbit erythrocyte membrane and formed oligomers that migrated in SDS-PAGE with an apparent molecular weight of 240,000 when incubated with SDS at 60°C (Fig. 1C). Insertion of the hemolysin in the membrane and subsequent oligomerization to an SDS-stable tetramer was also observed in PC-cholesterol (Fig. 1D, lane 6) as well as PC vesicles (Fig. 1D, lane 5). Since vesicles constituted from PC alone could effectively mimic the role of erythrocyte membrane in inducing oligomerization of the hemolysin, the process did not require participation of nonlipid constituents of the biomembrane. The interaction of hemolysin with immobilized cholesterol as determined by an ELISA-based assay (Fig. 1E) was significantly stronger than that with PC or phosphatidyl ethanolamine (PE). However, the extent of difference did not suggest a marked discrimination of polar head groups of the lipids by the protein.

Hemolysin Oligomer, a Nonlytic Lectin with Hemagglutinating Activity—The hemolysin oligomer formed in PC-cholesterol vesicles was isolated by solubilization of the protein-lipid complex in 1% sodium deoxycholate followed by gel filtration on Sepharose S-300 in the presence of the detergent. The oligomer, unlike the monomer that was stable indefinitely at 4°C, tended to precipitate in aqueous solution in storage and showed polydispersity during size exclusion chromatography on Sepharose CL-4B (not shown), indicating nonstoichiometric aggregation of the oligomers. The oligomer had no hemolytic activity toward rabbit erythrocytes at a concentration as high as 1 mg/ml. Absence of hemolytic activity of the protein corresponded with complete loss of ability to interact with immobilized PC, PE, or cholesterol (Fig. 1E). Interestingly, the hemolysin oligomer agglutinated rabbit erythrocytes strongly at a concentration of 10 ng/ml and human or sheep erythrocytes at about a 200-fold higher concentration.

Although monosaccharides such as mannose, glucose, galactose, l-fucose, N-acetylgalcosamine, and N-acetylgalactosamine were noninhibitory at a concentration of 100 mM, fetaun and calf thioglycolobulin, glycoproteins with multiple galactose-containing asparagine, and serine/threonine linked sugar units (25) inhibited the hemagglutinating activity of the oligomer (Table I). Ovalbumin or bovine pancreatic DNase, each of which contains a single oligomannosidic unit devoid of galactose, was noninhibitory. The oligomer showed erythrocyte species preference, although not very pronounced, with rabbit erythrocytes being 6- and 8-fold more sensitive than human and sheep cells, respectively.

Lectin-like Property of Hemolysin Monomer, Effect on Hemagglutinating Activity—Oligomerization of the hemolysin to a hemagglutinating lectin suggested that the monomer, although incapable of agglutinating erythrocytes by cross-linking opposing cells apparently due to lack of multivalency, might itself be endowed with carbohydrate-binding activity. The hemolysin

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; ELISA, enzyme-linked immunosorbent assay.
FIG. 1. A, percent hemolysis of rabbit erythrocytes as a function of hemolysin concentration. Erythrocyte suspensions (1% v/v) were incubated with various concentrations of the hemolysin for 60 min at 25 °C. B, kinetics of hemolysis of rabbit erythrocytes (1% v/v) as a function of hemolysin concentration. Symbols show percent decrease in turbidity (16) at a hemolysin concentration of 5 (○), 10 (●), 25 (△), 50 (▲), 100 (□), and 250 ng/ml (■). C, oligomerization of V. cholerae hemolysin in rabbit erythrocyte membrane detected by SDS-PAGE and immunoblotting. Rabbit erythrocytes were treated with the hemolysin suspension for 30 min to ensure complete lysis. The erythrocyte membrane was recovered by centrifugation at 20,000 g for 30 min at 4 °C, washed, and incubated in SDS at 30, 60, and 100 °C for 15 min. Following separation in 4–20% density gradient gel, the proteins were electrophoretically transferred (24) to nitrocellulose paper and visualized by immunoblot against rabbit anti-hemolysin antiserum (1:100). Membrane incubated with SDS at 30 °C for 15 min, lane 1; incubated at 60 °C for 15 min, lane 2; incubated at 100 °C for 5 min, lane 3. D, oligomerization of V. cholerae hemolysin in lipid vesicles without incorporated glycoconjugates. Proteins were run in 4–20% acrylamide gradient gel and visualized by staining in Coomassie Blue. Lane 1, low molecular weight standard proteins incubated with sample buffer containing 1% SDS at 100 °C for 5 min; lane 2, hemolysin (10 µg) incubated at 100 °C; lane 3, hemolysin incubated at 60 °C; lane 4, hemolysin in PC vesicle incubated at 100 °C; lane 5, hemolysin in PC vesicle incubated at 60 °C; lane 6, hemolysin in PC-cholesterol vesicle incubated at 60 °C; lane 7, high molecular weight standard proteins incubated at 60 °C for 15 min. E, interaction of monomeric and oligomeric forms of hemolysin with cholesterol, PC, and PE quantitated by ELISA. Each point represents an average of three independent determinations. Error bars indicate maximum dispersion from mean values. ●, ■, and ▲ represent binding of hemolysin to cholesterol, PC, and PE respectively; ○, □, and △ represent binding of hemolysin oligomer to cholesterol, PC, and PE respectively.
bound to asialofetuin with an association constant of $9.4 \times 10^7$ M$^{-1}$ as determined by the Scatchard plot (27) of the ratio of the bound to the free hemolysin against bound hemolysin (Fig. 2A) but not to ovalbumin or periodate-treated asialofetuin, which lack terminal $\alpha$-galactosyl moiety (not shown). Further, binding of the hemolysin to asialofetuin was partially reversed by preincubation of the protein with galactose and N-acetylgalactosamine but not by mannose (Fig. 2B). Pretreatment of immobilized asialofetuin with peanut agglutinin, a lectin specific for terminal $\alpha$-galactosyl moiety of glycoconjugates (18), led to a significant decrease in binding to the hemolysin (Fig. 2C). Concanavalin A (28) did not affect the binding of asialofetuin to the hemolysin under similar conditions.

Identification of the hemolysin as a lectin-like protein specific for glycoconjugates with terminal $\alpha$-galactosyl residues suggested that it might be capable of binding to erythrocyte surface carbohydrate receptors. In order to see whether this sugar-specific interaction was a prerequisite for induction of hemolysis the potential carbohydrate receptors for the hemolysin were blocked by the hemolysin oligomer as well as the peanut agglutinin. Although the time course of hemolysis was somewhat affected, as indicated by a marked shortening of the prelytic period, the equilibrium value of percent hemolysis was not affected (Fig. 3A) suggesting that binding of the hemolysin to carbohydrate receptors was not essential for insertion of the hemolysin under similar conditions.

Although hemolysis did not require binding to cell surface carbohydrate receptors, specific glycoproteins inhibited the hemolytic activity of V. cholerae hemolysin. Asialofetuin and asialothyroglobulin, but not ovalbumin or periodate-treated asialofetuin, caused a concentration-dependent decrease in the rate of lysis (Fig. 3C), indicating that inhibition of hemolysis involved specific binding of the hemolysin to $\alpha$-galactosyl moiety of glycoproteins. Since the hemolysin preincubated with asialofetuin migrated in Sephacryl S-300 as a lytically active monomer, the inhibition by glycoproteins did not arise from sugar-induced oligomerization of the hemolysin to lytically inactive aggregates.

The results seemed to imply that carbohydrates inhibited a process that did not require binding of the protein to carbohydrate receptors. That this was indeed so was clearly indicated by the inhibition of interaction of the hemolysin with immobilized PC by asialofetuin but not by periodate-treated asialofetuin (Fig. 3D). This result implied that glycoproteins with terminal $\alpha$-galactosyl moiety inactivated the hemolysin by inducing transition of the protein to a form incapable of interacting with lipid bilayers rather than by competing with cell surface carbohydrate receptors.
DISCUSSION

In this communication, we showed that *V. cholerae* hemolysin is a lectin-like protein that binds to glycoproteins with terminal β1-galactosyl moiety. As observed with other pore-forming cytolysins (10), the hemolytic activity of the protein depends on its interaction with membrane lipids that lead to formation of oligomeric pores by lateral collision in the bilayer plane. Specific glycoproteins inhibit *V. cholerae* hemolysin by inducing conversion of the protein to a form incapable of inserting itself in lipid bilayers rather than by competing with erythrocyte surface carbohydrate receptors. Inhibition of hemolysis by glycoproteins is, therefore, mechanistically different from that of well characterized lectin-induced cellular events like agglutination of erythrocytes and mitogenesis of lymphocytes (29). While the biological function of a lectin is usually a sequel to its ability to interact specifically with cell surface sugars, the sugar-binding property of *V. cholerae* hemolysin appears to be involved in regulating a function not dependent on interaction of the protein with sugars. A regulatory rather than functional role for carbohydrates might also resolve the apparent inconsistency in the finding that, although *S. aureus* α-toxin was inactivated by the erythrocyte membrane band 3 glycoprotein (14), the sensitivity of erythrocytes to lysis was not correlated with the amount of the band 3 glycoprotein in the membrane (30). A mechanistically similar regulation of the carbohydrate-independent interaction of concanavalin A with pure PC vesicles by methyl α-mannoside was reported earlier (31) and was attributed to a sugar-induced conformational change in the protein (32, 33).

The hemolysin oligomer that forms transmembrane diffusion channels in artificial lipid bilayers (8) and erythrocyte membrane (9) aggregated in water to a polydisperse mixture and adsorbed to phenyl-Sepharose matrix with an affinity that could be neutralized at an ethylene glycol concentration of 80% compared to 30% required for the desorption of the hemolysin monomer under similar conditions. Apparently, the presence of a lipid bilayer-spanning apolar domain in the hemolysin oligomer (10) contributed to the increased global hydrophobicity. However, the oligomer, in contrast to the mono-

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2 N. Saha and K. K. Banerjee, unpublished observation.
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mer, failed to insert itself in the erythrocyte membrane or to bind to immobilized phospholipids (Fig. 1F). These observations would suggest that a relatively small domain of the hemolysin monomer was involved in mediating the interaction of protein with lipids.

We have not characterized biochemically or genetically the site(s) of the hemolysin molecule involved in interaction with lipids and glycoproteins. They appear to be associated with distinct domains of the protein for the following reasons. (i) Oligomerization of the monomer abolished lipid-binding activity without essentially affecting the sugar-binding property, and (ii) it is unlikely from considerations of steric complementarity as well as types of noncovalent forces involved in binding of protein to sugars and lipids for the same molecular domain of the protein to interact with these two classes of compounds. These considerations suggest that lipids bind to the active or functional site of the hemolysin and glycoproteins to the regulatory site.

Two classes of rabbit erythrocyte surface sites may likewise be distinguished, pore formation or hemolysis sites represented by exposed patches of phospholipid-cholesterol bilayer and agglutination sites represented by sugar moieties of glycoproteins or glycolipids. While the oligomeric form of the hemolysin binds to carbohydrate receptors only leading to agglutination, the monomeric form of the hemolysin is potentially capable of interacting with both these sites. Glycoprotein-induced conversion of the hemolysin to a lytically inactive form incapable of interacting with lipid bilayers suggested that cell surface sugars might contribute to the insensitivity of erythrocytes to the hemolysin by causing a decrease in the effective concentration of the hemolysin available for productive interaction with phospholipid cholesterol bilayer leading to pore formation. This contention was corroborated by the inverse correlation between susceptibility of rabbit erythrocytes to V. cholerae hemolysin and the surface density of glycoconjugates specifically recognized by the hemolysin (Fig. 3B). Similar considerations may explain the decrease in the rate of insertion of S. aureus a-toxin in phospholipid-GM1-ganglioside liposome with increasing glycolipid content (34). Although pore-formation process is facilitated by a high concentration of the inserted monomer in the bilayer plane (Fig. 1B), kinetics of the overall process and especially the length of the lag phase are regulated primarily by membrane events that include diffusion of the monomer in the bilayer plane and self-aggregation by lateral collision (10, 12). These events are highly sensitive to parameters that affect the property of the membrane bilayer matrix as a diffusional medium (12). It appears, therefore, that the shortening of the prelytic period of hemolysis on preincubation of erythrocytes with lectins (Fig. 3A) was due to perturbation of the membrane bilayer induced by cross-linking of glycoconjugate receptors by multivalent lectins (29), rather than due to suppression of an abortive interaction of the hemolysin with sugars.

Biological relevance of the sugar-binding property of V. cholerae hemolysin has not been considered in this communication. Recent reports suggest that the lectin-like function of a protein does not necessarily determine its interaction with cell surface. For example, discoidin I, a galacose-specific lectin of cellular slime mold, binds to cells by a carbohydrate-independent mechanism similar to those mediated by fibronectin (35). The sugar-binding function of the protein is, however, involved in correct localization and exocytosis of the protein in the presence of specific oligosaccharides (36). Export of proteins by Gram-negative bacteria to the extracellular milieu requires passage through lipid barriers of the outer and inner membranes. Since V. cholerae hemolysin is excreted in a monomeric, lytically active form, it must have evolved a mechanism to arrest premature self-aggregation to a lytically inactive form during exposure to lipid bilayers of bacterial membranes. This is, perhaps, true of pore-forming cytolsins in general. Recently, it has been found by genetic studies that an oligosaccharide sequence of the lipopolysaccharide mediates prevention of premature aggregation of Escherichia coli α-hemolysin (37), suggesting that specific bacterial glycoconjugates might mimic the role of molecular chaperones (38). Evidence from genetic studies also indicates that the membrane insertion and trimerization behavior of E. coli porins (39) and the localization of IcsA, an outer membrane protein responsible for intracellular spread of Shigella flexneri (40), are regulated, by an unknown mechanism, by the oligosaccharide moiety of lipopolysaccharide. The observations suggest that these outer membrane proteins may have lectin-like functions that might serve, in the presence of appropriate glycoconjugates, as information necessary for targeting them to the correct destinations. Further study may elucidate the biological significance of sugar-binding motif of a protein that does not appear to be designed to mediate its cognitive interaction with cell surface carbohydrates.

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