Biochimica et Biophysica Acta, 775 (1984) 273–282
Elsevier

BBA 72169

FREEZE-ETCH STUDY OF AN UNMODIFIED LECTIN INTERACTING WITH ITS RECEPTORS IN MODEL MEMBRANES

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(Received January 3rd, 1984)

Key words: Lectin receptor; Membrane-lectin interaction; Freeze-etch electron microscopy; (Model membrane)

(1) Experiments are described in which liposomes bearing ganglioside or glycophorin as receptor were exposed to the native (unmodified) lectin, wheat germ agglutinin, and subsequently examined by freeze-etch electron microscopy. Visualized in this way in the absence of lectin, phosphatidylcholine bilayer membranes show no features attributable to the presence of small amounts of glycolipid. Similarly bilayers bearing glycophorin show no obvious etch face (outer surface) features attributable to that species, although they do have intramembranous particles associated with the hydrophobic interior. However, the otherwise smooth and featureless model membrane outer surface permitted ready visualization of bound lectin molecules, and thus the indirect localization of receptors. (2) The lipid membrane itself in the vicinity of receptors was not visibly affected by lectin binding to glycolipid or glycoprotein. The only identifiable lectin-induced change was lateral redistribution of receptor glycoproteins. Ganglioside molecules showed some evidence of existing in small clusters; but their distribution was apparently unaffected by lectin binding. (3) Clumps of lectin bound to glycophorin were found associated predominantly with fluid bilayer regions of phase separated membranes; and lectin distribution on the etch face correlated with intramembranous particle distribution in the fracture face. In contrast, ganglioside lateral distribution was not appreciably influenced by the host lipid matrix phase separation.

Introduction

Glycosphingolipids and glycoproteins have attracted considerable attention in recent years. In part the interest stems from their demonstrated role as recognition sites for agents of the immune system, lectins, and polypeptide hormones and toxins. There is, in addition, their postulated involvement in adhesion, tissue formation, differentiation, and oncogenic transformation. The major impetus in our laboratory has been toward understanding the molecular basis of receptor behaviour, and possible modification of such behaviour by specific binding events. An attractive approach to this area of research is to use lipid bilayer model membranes, since under optimal circumstances they provide a controllable level of complexity. A variety of techniques have been applied to the study of lectin/receptor interactions: including analytical quantitation of the event, and spectroscopic probe studies. In this article we describe the application of freeze-etch electron microscopy to the problem, an approach which permits actual visualization of the lectin-receptor complex.

Freeze-etching is a shadowing technique which

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Abbreviations: DEPC, dielaidoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; RCA, *Ricinus communis* agglutinin.

offers extensive face views of membrane architecture, resolving details down to some 3–4 nm. It also has the unique ability to demonstrate features in the hydrophobic interior. The membrane specimen is rapidly frozen ('quenched') from some temperature of interest, fractured in vacuum to expose membrane hydrophobic regions, etched in vacuum to expose membrane surfaces by sublimation of surrounding ice, and then coated with an atomic beam of platinum. When prepared in this way, cell membrane fracture faces appear as smooth surfaces interrupted by well-defined intramembranous particles [1] representing points of membrane penetration by protein. The etch faces of cell plasma membranes are typically smooth and featureless [2] but upon close examination may show an indistinct granularity associated with their very extensive surface protein. Simple lipid bilayers studied by freeze-etching are characterized by smooth fracture and etch faces, reflecting the lack of protruding structure in these domains. Incorporation of integral proteins into such bilayers gives rise to (hydrophobic interior) intramembranous particles, but as a rule any resultant surface alterations are not well visualized when coated with platinum. Glycolipids incorporated into lipid bilayer membranes at low mol ratios give rise to no identifiable features. We have found that it is possible, because of the otherwise smooth appearance of lipid bilayer etch faces, to resolve native lectin bound to glycolipid and glycoprotein receptors in model membranes.

The receptors chosen for this work were the glycoprotein, glycophorin, and the neuraminic-acid bearing family of glycosphingolipids, gangliosides. The former is the wheat germ agglutinin receptor in human erythrocytes as well as the MN blood group determinant (Refs. 3 and 4, and references therein); and is widely accepted as an example of a glycoprotein which traverses the cell membrane. Glycophorin has an M_r of some 30000 and bears 16 short oligosaccharide chains of which 15 are O-linked. Wheat germ agglutinin, the lectin used in this work, is a 36000 M_r species composed of two similar polypeptide chains, each possessing two sugar binding sites [5,6]. It binds to *N*-acetylglucosamine and also to *N*-acetylneuraminic acid (Refs. 5, 7 and 8 and references therein). The techniques used for incorporating glycophorin and gangliosides into lipid bilayer model membranes have been described previously [9–11]. Glycolipids are readily incorporated into lipid bilayers by simply dissolving them with phospholipids in organic solvent, removing solvent by evaporation, and hydrating the thin films so formed. The method chosen to assemble glycophorin into bilayers involved detergent solubilization of lipid plus protein and subsequent exhaustive dialysis.

Materials and Methods

DMPC, DPPC, cholesterol, wheat germ agglutinin (WGA) and RCA 60 were obtained from Sigma. DEPC was from PL Biochemicals. Bovine brain ganglioside was isolated using a modification of the method of Kanfer [12] in which ganglioside obtained from the initial Folch extraction was purified by chromatography on silicic acid (Bio-Rad, 200–400 mesh, eluting with $CHCl_3$/CH_3OH). G_{M1} and G_{D1a} were from Supelco. All lipids were pure as judged by thin-layer chromatography on plates coated with silica gel GF_{254} (Stahl). Glycophorin was isolated from human erythrocyte ghosts according to the method of Marchesi and Andrews [13].

Liposome preparation. Glycophorin was incorporated at 11% by weight (0.32 mol%) into liposomes of 1 : 1 (mol ratio) DEPC/DPPC by dissolving all components in 300 mM dodecyltrimethylammonium bromide, and subsequent exhaustive dialysis at 4 °C against 10 mM phosphate buffer pH 7.4 [9]. The detergent was purchased from Sigma and recrystallized from acetone/methanol. Gangliosides were incorporated at 7 mol% into liposomes of 1 : 1 (mol ratio) DEPC/DPPC by mixing all lipids in chloroform/methanol and drying as a thin film under nitrogen. Films were further dried in vacuum and then hydrated with 10 mM phosphate buffer (pH 7.4) at 41 °C followed by vortexing gently with glass beads. All liposomes were warmed to 45 °C and allowed to cool slowly to 20 °C prior to preparation for freeze-etching. Samples for freeze-etching were suspended at a concentration of 0.33 mg per ml total lipid and incubated with various concentrations of lectin for 15 min at 20 °C. Liposomes were then quickly harvested by centrifugation at

$120 \times g$ and frozen in freon cooled in liquid nitrogen.

Freeze-etch replicas were prepared by platinum shadowing in a Balzers apparatus. Samples to be directly compared were handled simultaneously to avoid any differences due to shadow variability. Etching was for 2 min at $-103°C$. Replicas were cleaned in $NaClO_4$, rinsed with distilled water, and then exposed to acetone to remove traces of lipid. Replicas were examined using a Phillips EM300.

Results and Discussion

Visualization of the lectin-glycoprotein complex

Fig. 1 illustrates the appearance of liposomes bearing glycophorin as receptor and exposed to various concentrations of wheat germ agglutinin. The host phospholipid matrix was 1 : 1 (mol ratio) DPPC/DEPC. Pure, fully hydrated bilayers of the C-18 (unsaturated fatty acid) phospholipid, DEPC, undergo a sharp phase transition between rigid (gel phase) and fluid (liquid-crystal phase) states at 1°C [13]. Bilayers of the C16 (saturated fatty acid) phospholipid, DPPC, have a phase transition temperature of 41.5°C [15,16]. Bilayers formed of mixtures of these two pure lipids have a phase behaviour best thought of in terms of the phase separation phenomenon, originally applied to membranes by Chapman [17] and McConnell [16] and co-workers (see also Refs. 18 and 19). One result of this behaviour is that at intermediate temperatures, phase separated (laterally distinct) regions of different composition and properties coexist in dynamic equilibrium. This property has certain attractive features for the present study in that it increases the information content and interpretability of the results, and perhaps better mimics the proposed existence of lipid microdomains in cell membranes than would a single pure phospholipid. In this particular lipid mixture in the temperature region of coexisting rigid (enriched in DPPC) and fluid(enriched in DEPC) domains, the former is visually identifiable [14,19,20] by a ripple pattern characteristic of the $P_{\beta'}$ phase of Tardieu et al. [21]. This feature is evident in all of the micrographs shown in Fig. 1 since each sample was 'quenched' (rapidly frozen in liquid Freon) from a temperature of 20°C. The phase diagram for this lipid mixture at 20°C would dictate that a given liposome should contain roughly equal amounts of rigid lipid with a composition of some 1 : 3 DEPC/DPPC and fluid lipid with a composition 3 : 1 DEPC/DPPC [14].

Thus Fig. 1A shows a liposome such as described above, bearing 11 wt% glycophorin but not exposed to wheat germ agglutinin. The specimen was quenched rapidly from 20°C, fractured to expose the hydrophobic membrane interior (right hand portion of figure) and then etched in vacuum to sublime away surrounding ice and thus expose the membrane outer surface (smooth band extending from top to bottom). The surrounding ice may be seen on the left. Note that the etch face (outer surface) is largely featureless with only faint granularity perhaps attributable to glycoprotein headgroups. The lower portions of both fracture face and etch face show linear markings demarcating the rigid ('rippled') $P_{\beta'}$ phase coexisting with more fluid lipid in the upper portion of the membrane. The fracture face shows well resolved intramembranous particles (IMP) characteristic of proteins penetrating a hydrophobic membrane interior [1]. Such particles in model membranes bearing glycophorin were first reported by one of us in H.M. McConnell's laboratory [9]. It was suggested that they represented small clusters of the glycoprotein since they are larger than anticipated, their size and shape are somewhat irregular, and since in optimal replicas they can often be resolved into groups of several smaller particles. Similar conclusions have been reached in this laboratory and others since [22–24]. It is very possible that monomeric glycophorin may give rise to a membrane feature so small as to be undefined in many freeze-etch preparations (see Ref. 24). There is also a pitting effect in the fluid region of the fracture face which is often seen in protein-containing model membranes which have been deep etched. In other words, dispersed amongst the glycophorin intramembranous particles which protrude from the fracture face as irregular bumps 10–20 nm in diameter, depressions of similar dimensions may be found. The origin of such pits is poorly understood, although they may represent the corresponding defects left by proteins torn away during fracture removal of the overlying lipid monolayer. Note however, that there are no

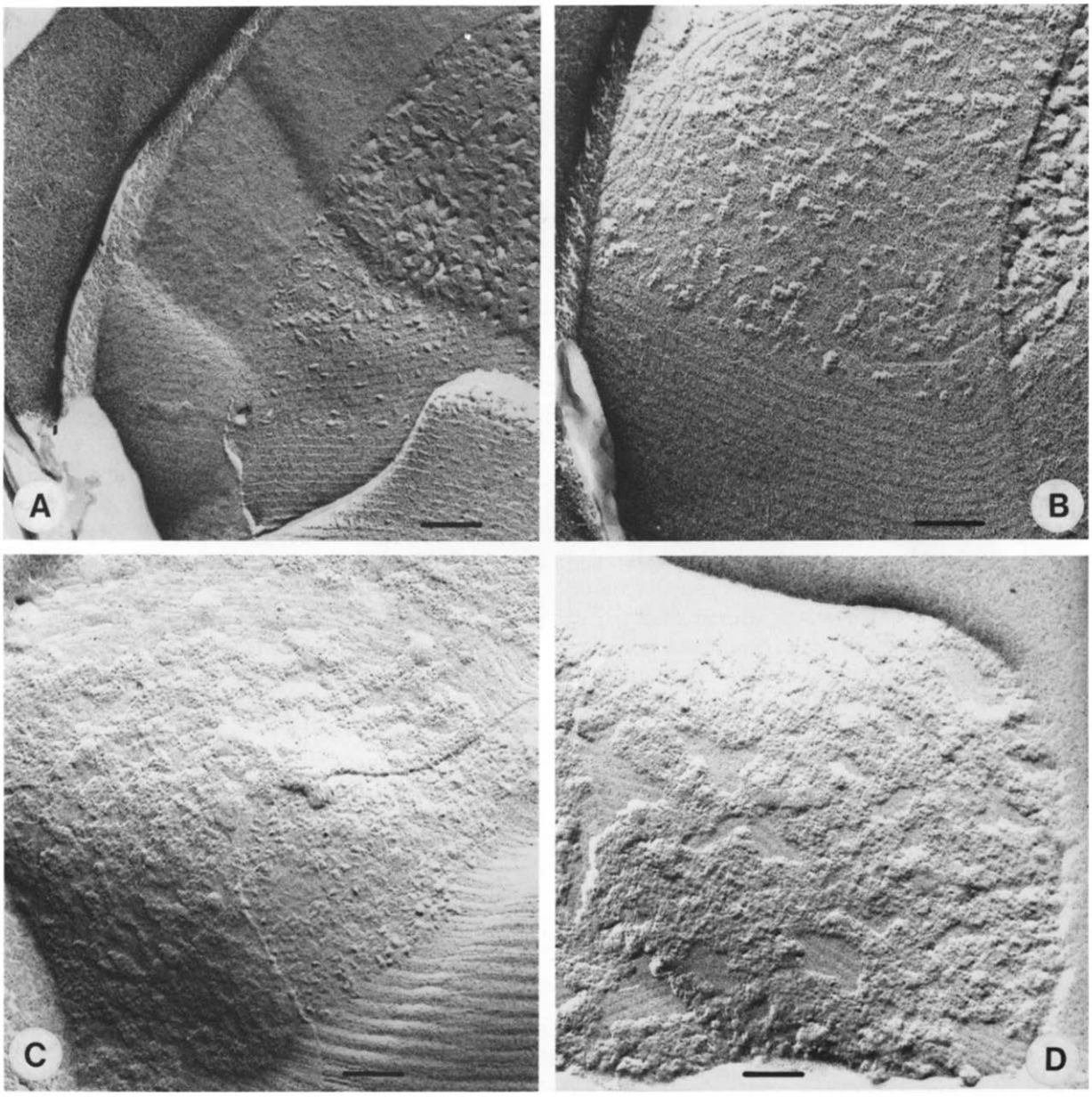

Fig. 1. Freeze-etch electron micrographs of liposomes bearing the integral membrane glycoprotein, glycophorin, which have been exposed to various concentrations of wheat germ agglutinin. In each case the lipid bilayer host matrix of 1:1 (mol ratio) DEPC/DPPC contained 0.32 mol% glycophorin, and specimens were quenched (rapidly frozen) from 20 °C. Under the sample conditions described, the lipid bilayers may be seen to possess coexisting, (laterally) phase separated rigid and fluid domains in roughly equal proportion. The 'ripple' pattern marks regions of rigid $P_{\beta'}$ phase selectively enriched in the higher melting DPPC (see text). Immediately prior to quenching, the liposomes were exposed for 15 min to wheat germ agglutinin at the following concentrations; A, 0 mg/ml; B, 0.01 mg/ml; C, 0.1 mg/ml; D, 1 mg/ml. Shadow direction is from bottom to top of page. Bar denotes 0.1 μm.

grossly obvious features on the membrane etch face which one could attribute to the glycophorin molecule. Apparently the portion external to the membrane is too insubstantial to give rise to a sharp platinum shadow.

It is immediately clear that the lateral distribution of glycophorin-related intramembranous particles is influenced by the nature (fluid vs. rigid) of the bilayer host matrix (as has been reported previously [9,22,24]). There is a strong tendency to exclusion of intramembranous particles from the rigid membrane regions which may be adequately predicted on the basis of a theoretical treatment which considers the integral protein as an impurity in an ordered crystal (lipid) lattice [9,25–28]. This phenomenon has also been reported for several other proteins in model membranes, including rhodopsin [29], band 3 [22], and the (Ca^{2+} + Mg^{2+})-ATPase from rabbit sarcoplasmic reticulum [25].

Figs. 1B–D illustrate the appearance of liposomes similar to that already described, but exposed to increasing concentrations of the unmodified tetravalent lectin, wheat germ agglutinin, for 15 min immediately prior to quenching in Freon from 20 °C. In Fig. 1B an extensive region of etch face is shown (narrow strip of ice on left, fracture face on right). The lower portion of the etch face bears the familiar rippled pattern of rigid lipid, while the upper portion is a coexisting fluid domain (becoming rigid again in the upper left portion). Clearly there is a granular pattern of bound lectin on the outer surface of the fluid membrane domain. The granularity occurs in small irregular piles some 30 nm across. Since wheat germ agglutinin is a dimer of M_r 36 000 one would expect it to be at the limit of resolution of the freeze-etch technique with a diameter of roughly 4–6 nm when coated with platinum. The irregular piles seen must represent numerous wheat germ agglutinin molecules bound to glycophorin receptors. In this regard note that each glycophorin molecule has a headgroup region which consists of a flexible polypeptide chain some 15 nm long bearing 16 oligosaccharide chains, each of which can bind wheat germ agglutinin. In Figs. 1C, D the lectin concentration was considerably higher: 1 mg/ml. In both cases the bound lectin forms extensive mats on the liposome etch faces.

Note that Figs. 1B–D show that the receptor (glycophorin) distribution marked by bound lectin on the etch face is exactly that anticipated on the basis of intramembranous particle distribution in the hydrophobic membrane interior. This is not a trivial expectation since, as described above, on the basis of its low molecular weight monomeric glycophorin species would not be expected to show up well in the fracture face and might not exist in the same distribution as clusters of glycophorin. This was a problem that had concerned us since our initial studies based on fracture face observations.

Effects of lectin binding to receptor glycoproteins

An appealing concept is to think of receptors in membranes as delicately balanced triggers which, when specifically stimulated by various external contact events, are capable of initiating complex responses on the part of the cell. The actual triggering process could presumably be a lateral rearrangement of receptor, a perturbation of the receptor or its environment, or internalization of the receptor/ligand. In model membranes there is the potential for focussing on individual factors in an attempt to unravel their molecular bases. The ability to actually visualize receptors at high magnification should be very beneficial in this pursuit.

There are several aspects of the receptor/membrane system that one might examine for evidence of a lectin-mediated alteration. Firstly, the receptor itself is visible as an intramembranous particle in the fracture face, and at the resolution possible with the freeze-etch technique one can certainly visualize structural detail in these intramembranous particles. It has been commented previously that in good quality replicas the glycophorin-related intramembranous particles often appear to be small groups of particles, in keeping with the idea that glycophorin exists as clusters in model membranes. We have not been able to describe any clear alteration in intramembranous particle structure upon lectin binding (e.g., comparing the fracture face in Fig. 1A to those in Figs 1B and 1C). That is, their size, shape and detailed appearance remain unaltered upon lectin binding. Having stated this though, we recognize that such details are very much at the limit of resolution of

the freeze-etch technique so that one cannot rule out the presence of small changes. The second feature of the membrane that one might examine for changes is the appearance of the lipid bilayer in close association with bound receptor (etch face or fracture face). Once again there is no apparent alteration following lectin binding. In this regard note for instance that in Fig. 1B the linear ripple pattern may be seen to pass underneath certain patches of lectin-receptor complex (upper left region of etch face) with no alteration in direction or position. These lines reflect orderly sequential lipid packing analogous to the linear growth of an ice crystal, and at the junctions of fluid/rigid domains would be affected by any gross membrane perturbation. We have never seen evidence of such an effect. Thirdly, there is the question of receptor distribution, and this does seem to be measurably altered by lectin binding. Lectins are multidentate molecules with a potential for receptor crosslinking. Our laboratory has reported previously that fluorescent lectins can induce a patchiness to the distribution of glycophorin in fluid bilayer membranes [30]. With electron microscopy one may view this phenomenon in greater detail. Fig. 1C in particular illustrates both in fracture and etch face a moderate patchiness to receptor distribution with smooth areas in between. The lateral translocation induced by WGA in this system is on the order of tens of nm in a 15 min period.

Visualization of the lectin-glycolipid complex

Using the same binary mixture of phospholipids (1 : 1 DEPC/DPPC) as host matrix, we have examined the binding of native wheat germ agglutinin to ganglioside in model membranes. Membranes bearing 7 mol% ganglioside (from bovine brain) show no obvious features directly attributable to the presence of the glycolipid. the headgroup is too small to cast a platinum shadow on the etch face, and there is no polypeptide portion penetrating the hydrophobic region to give rise to an intramembranous particle. These observations are demonstrated in Fig. 2A which shows an extensive region of liposome etch face (fracture face on right) which was exposed to wheat germ agglutinin in the presence of inhibitory sugar. In contrast Fig. 2B shows the etch face of a liposome exposed to wheat germ agglutinin in the absence of inhibitory sugar. The etch face has a marked labelling with bound lectin. The actual visualization of small molecules at membrane surfaces is optimized in regions where the platinum shadow impinges at a low angle to the plane of the membrane; although at very low angles detail is lost. By working with a very clean vacuum system we have avoided problems of artefact generation by water and oil vapour.

Figs. 2C–E are micrographs chosen because they show rippled (rigid) domains. In C and D these are clearly in coexistence with smooth regions of bilayer. Certainly there is no obvious exclusion of (lectin-labelled) glycosphingolipid from rigid domains as there was in the case of the glycoprotein already discussed. Counting of (granular) lectin deposits on rippled regions for comparison with neighbouring smooth regions shows no significant difference in occupancy. Note that none of the micrographs show any evidence of a correlation between ripple pattern and lectin pattern. That is, there is no evidence of a special alignment or distribution relative to the ripple pattern.

Effects of lectin binding to receptor glycolipids

Since glycolipids themselves do not give rise to identifiable membrane features in freeze-etched preparations, it is not possible to look for lectin-related changes in such features as it was in the case of glycoprotein intramembranous particles. It is of course possible to examine the membrane hydrophobic interior and external surface for visible alterations. One might anticipate that with the lectin binding site (oligosaccharide chain) so intimately attached to the membrane surface, glycolipid-bearing samples would be more likely to show a local lipid effect of lectin binding than glycoprotein-bearing ones. In fact, there is no such effect observed. Once again note that there is no alteration of ripple pattern at the labile boundaries of rigid and fluid domains.

Unmodified wheat germ agglutinin is a globular species of only 36 000 M_r which can be expected to produce at best a small etch face 'bump'. On this basis, many of the granular particles seen on the etch face must be clumps of several or more lectin. A given ganglioside may have more than one terminal sugar capable of binding wheat germ

agglutinin (e.g. *N*-acetylneuraminic acid in G_{D1a}). Hence there exists the possibility that such clusters might represent a single glycolipid with several bound lectin molecules and/or clusters produced by headgroup crosslinking. A consideration of space-filling molecular models combined with the

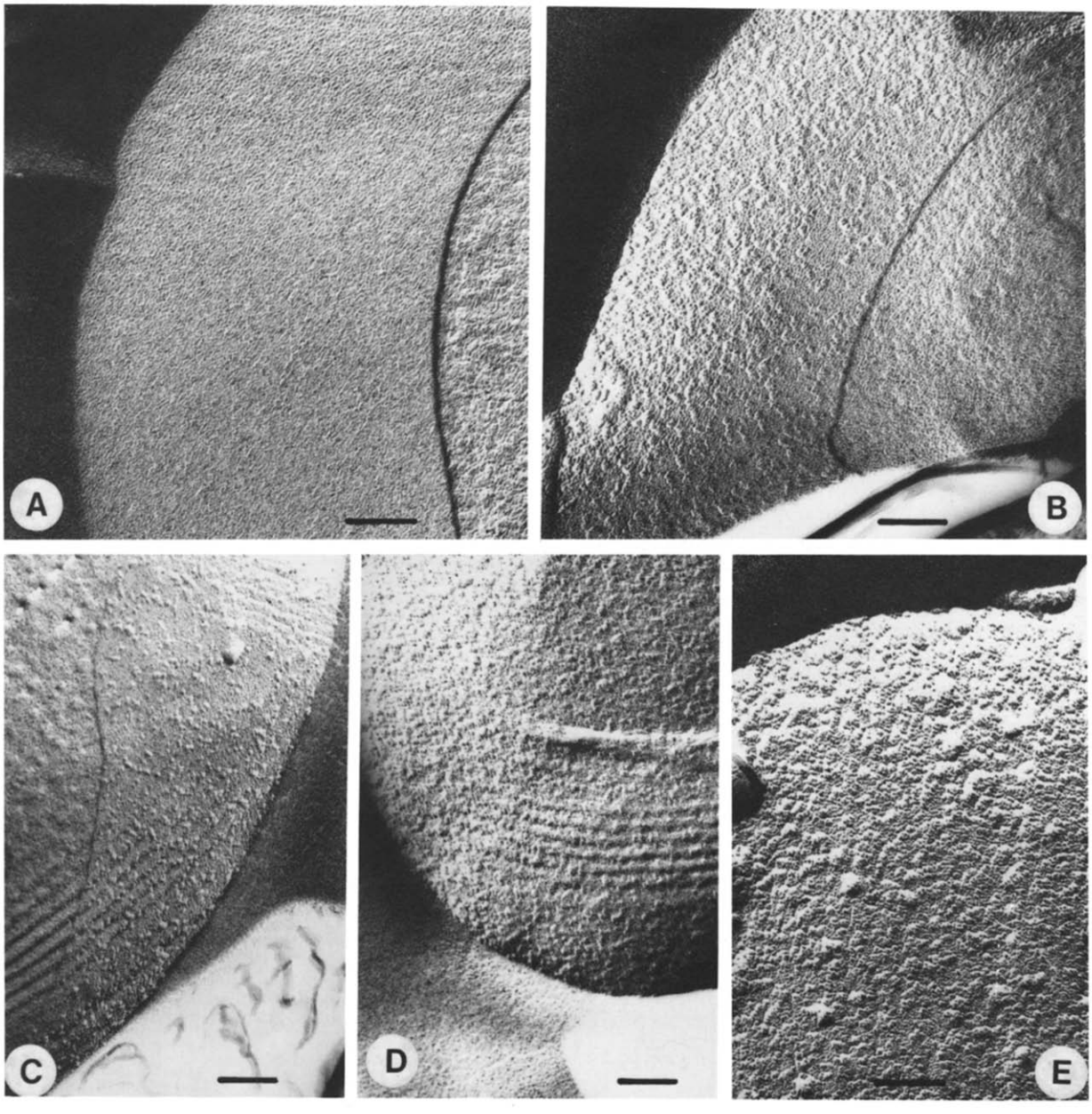

Fig. 2. Freeze-etch electron micrographs of liposomes bearing 7 mol% bovine brain ganglioside which have been exposed to various concentrations of the lectin, wheat germ agglutinin. In each case the lipid bilayer host matrix was composed of 1:1 (mol ratio) DEPC/DPPC, and liposomes were quenched for freeze-etching from 20 °C. As in Fig. 1, under these conditions coexisting membrane regions of rigid ('rippled') and fluid (smooth) lipid may be identified (see text). Prior to rapid freezing, samples shown were exposed for 15 min to wheat germ agglutinin at the following concentrations: A, 1 mg/ml in the presence of 0.2 M *N*-acetylglucosamine; B, D, E, 1 mg/ml; C, 0.1 mg/ml. Shadow direction is from bottom to top. Bar denotes 0.1 μm.

known critical orientational requirements for lectin/sugar binding suggests that such multiple occupancy of a sugar headgroup is sterically unlikely (see also Ref. 31). Probably then the surface features seen do not reflect lectin-induced redistribution. One may test this by considering liposomes with both fluid and rigid regions as was done here. In rigid bilayers the rate of lipid lateral diffusion is over 1000-times slower than in fluid bilayers (Ref. 32 and references therein); yet the etch face particle pattern is very similar in both types of region. Hence there is no evidence of receptor redistribution upon lectin binding in the case of our glycolipids.

Use of native lectins to localize glycolipids and glycoproteins in model membrane

The conventional approach to visualization of the topographic distribution for a given membrane species is to mark it with a labelled antibody or lectin. Typical examples of labels used for electron microscopy are 600000 M_r (iron dense) ferritin, and the enzyme, lactoperoxidase, which precipitates about itself an electron-dense deposit. We have previously used lectins linked to these labels successfully to examine the distribution of several glycoproteins in model membranes [33]. The unmodified lectin offers a more subtle marker in that it has no attached massive structure to be sterically influenced by surface conditions or to obscure detail; and it requires no modification of the lectin. On the other hand, small lectins such as wheat germ agglutinin are correspondingly less visually distinct than say their ferritin-conjugated analogues.

Relatively little is known about the lateral distribution, or factors that control lateral distributions, of glycolipids in membranes. An outstanding article from the laboratories of Tillack and Thompson describes the use of ferritin-conjugated ricin to pick out the distribution of the neutral glycolipid, asialo-G_{M1}, in bilayer membranes composed of single pure phospholipids (Ref. 31, see also Ref. 34). They found by

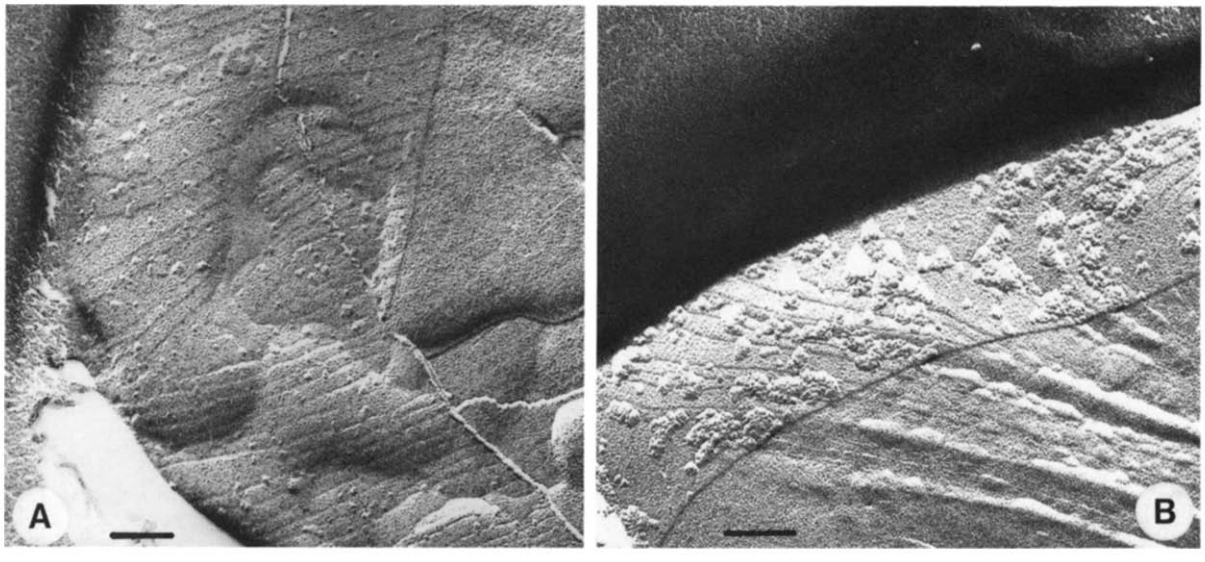

Fig. 3. Potential of native lectins as markers of receptor distribution. Freeze-etch electron micrographs of liposomes bearing the ganglioside G_{D1a} at 7 mol% in pure DMPC (A) and G_{M1} at 7 mol% in pure DPPC (B). In each case the samples were quenched from temperatures well below the host matrix phase transition temperatures of 23 °C for DMPC and 41.5 °C for DPPC [16]. The membrane suspension studied in Fig. 3A was exposed for 15 min at 4 °C to the 34000 M_r lectin, wheat germ agglutinin, prior to quenching from the same temperature. The membranes in (B) were exposed to the 60000 M_r species, RCA 60, at 20 °C prior to quenching from the same temperature. Both samples show the $P_{\beta'}$ ripple pattern referred to in the text and characteristic of a rigid host matrix. Clearly native RCA 60 is better resolved than the lower molecular weight wheat germ agglutinin as individual particles some 10 nm in diameter when coated with platinum and carbon. The clustered appearance of bound RCA 60 is quite striking. In each case shadow direction is from bottom to top, and the bar denotes 0.1 μm.

freeze-etch electron microscopy that at 7 mol% the glycolipid tended to exist in small clusters binding up to a dozen lectin/ferritin conjugates. The clustering phenomenon was seen in fluid as well as rigid membranes. The suggestion of glycolipid clustering in bilayer membranes has been made previously based on experiments with spectroscopic problems [35–39]. Another striking feature of the paper by Tillack et al. [34] is the appearance of linear arrays of ricin/ferritin conjugates aligned parallel to the ripple phase of rigid bilayers of single pure phospholipids (localized to the 'trough' region of the ripple patterns). In the work described here the distribution of beef brain ganglioside, as marked by native wheat germ agglutinin, is consistent with a tendency for them to exist in small clusters (see also Ref. 39). However, in our two-component lipid systems we have seen no evidence (Fig. 2) of the linear arrays described above. To test this further we examined the binding of wheat germ agglutinin to bilayers of pure DMPC bearing 7 mol% ganglioside, since DMPC as host matrix was reported to show this phenomenon strikingly [31]. For instance Fig. 3A shows the appearance of DMPC bilayers bearing G_{D1a} and exposed to wheat germ agglutinin. There is no obvious evidence of the linear arrays described by Tillack et al. [31]. Perhaps this reflects different behaviour of different glycolipid families, a phenomenon which warrants further experiment.

Although wheat germ agglutinin is convenient for freeze-etch studies involving sialic acid-bearing glycolipids and glycoproteins, there is no reason to expect it to be superior to other lectins for studies of receptor distribution in general. In fact its small size makes it difficult to resolve on membrane surfaces. Hence we have begun to experiment with other species; and in this vein Fig. 3B shows the monovalent RCA 60 from *Ricinus communis* bound to pure G_{M1} in (rigid) bilayers of DPPC at 20 °C. Note that RCA 60 with an M_r of 60000 [40] shows up more clearly than wheat germ agglutinin (as particles some 10 nm in diameter when shadowed with Pt/C). Clearly, bound RCA 60 occurs in clusters on the membrane surface, presumably marking the presence of underlying glycolipid clusters. As in the case of wheat germ agglutinin, lectin binding has no identifiable effect on the membrane itself. It is interesting to compare this example of native RCA 60 bound to 7 mol% G_{m1} in DPPC with a micrograph in Ref. 31 showing ferritin-conjugated RCA 60 bound to 7 mol% asialo-G_{M1} in DPPC: the appearance is very similar. It seems reasonable then to continue to employ native lectins as visual freeze-etch markers of receptor location where resolution permits. It is encouraging that there seems to be at least basic agreement with studies utilizing probe-labelled lectins.

Acknowledgements

This research was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada. M.W.P. was the recipient of an MRC studentship.

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