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Low concentrations of diacylglycerol promote the binding of apolipophorin III to a phospholipid bilayer: A surface plasmon resonance spectroscopy study

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ABSTRACT **The binding of the exchangeable apolipoprotein apolipophorin III (apoLp-III) to an egg phosphatidylcholine bilayer as a function of the concentration of diacylglycerol (DG) in the bilayer was studied by surface plasmon resonance spectroscopy. At a DG concentration of 2 mol % in the bilayer, the binding of apoLp-III reached saturation. Under saturating conditions, apoLp-III forms a closely packed monolayer ≈55 Å thick, in which each molecule of protein occupies ≈500 Å^2 at the membrane surface. These dimensions are consistent with the molecular size of the apoLp-III molecule determined by x-ray crystallography, if apoLp-III binds to the bilayer with the long axis of the apoLp-III normal to the membrane surface. In the absence of protein, the overall structure of the lipid bilayer was not significantly changed up to 2.5 mol % DG. However, at 4 and 6 mol % DG, the presence of nonbilayer structures was observed. The addition of apoLp-III to a membrane containing 6 mol % DG promoted the formation of large lipid–protein complexes. These data support a two-step sequential binding mechanism for binding of apoLp-III to a lipid surface. The first step is a recognition process, consisting of the adsorption of apoLp-III to a nascent hydrophobic defect in the phospholipid bilayer caused by the presence of DG. This recognition process might depend on the presence of a hydrophobic sensor located at one of the ends of the long axis of the apoLp-III molecule but would be consolidated through H-bond and electrostatic interactions. Once primary binding is achieved, subsequent enlargement of the hydrophobic defect in the lipid surface would trigger the unfolding of the apolipoprotein and binding via the amphipathic α-helices. This two-step sequential binding mechanism could be a general mechanism for all exchangeable apolipoproteins. A possible physiological role of the ability of apoLp-III to bind to lipid structures in two orientations is also proposed.**

Lipophorin is the main lipoprotein found in the hemolymph of insects; it transports phospholipid, diacylglycerol (DG), and hydrocarbons among insect tissues. The lipophorin particle contains between 35% and 65% (weight %) lipid and two nonexchangeable apolipoprotein molecules—one apolipophorin I molecule of ≈250 kDa and one apolipophorin II molecule of ≈80 kDa (1–5). In those lipophorin particles containing >35% lipid, which is mainly due to an increase in the DG content, a third molecule, the exchangeable apolipoprotein apolipophorin III (apoLp-III; ≈18 kDa), is also found. The number of molecules of apoLp-III bound to the lipophorin is related to the DG content and varies between 0 and at least 16 molecules. The interaction of apoLp-III with lipid is of interest for several reasons. (*i*) apoLp-III plays an essential role in the transport of large amounts of DG, which is the major source of energy for flight in many insects (1–5). (*ii*) apoLp-III is the only full-length exchangeable apolipoprotein whose structure has been determined by x-ray crystallography (6). The knowledge of the crystal structure of apoLp-III offers the possibility of relating the structure of an apolipoprotein to its function. (*iii*) The similarity between the properties of the insect and vertebrate exchangeable apolipoproteins (7) suggests that elucidation of the mechanism of interaction of apoLp-III with lipids would be directly applicable to vertebrate apolipoproteins.

In the present communication, we report the use of surface plasmon resonance (SPR) spectroscopy to investigate the mechanism of binding of *Manduca sexta* apoLp-III to a supported lipid bilayer and the effect of low DG concentrations on the lipid–protein interaction. SPR spectroscopy is a very sensitive technique for determining changes in the thickness and optical properties of thin films (8). SPR spectroscopy has recently been used to characterize the assembly of lipid monolayers and bilayers on gold or silver surfaces (9–11) and to study the interaction of several protein–ligand complexes—e.g., streptavidin–biotin (12–15), antigen–antibody (16), cholera toxin–ganglioside (17), and lactose repressor–DNA (18) complexes and the conformational changes induced by light in bovine rhodopsin (19).

MATERIALS AND METHODS

SPR Spectroscopy. Steady-state SPR measurements were performed by using the attenuated total reflection technique with the Kretschmann configuration and a thin silver film evaporated onto the base of a BK7 prism (refractive index, n = 1.515 at 632.8 nm), as reported (10, 19). The spectra were recorded by varying the incident angle of the laser beam, which was measured with an accuracy of ±0.01°. The spectra were recorded over a 20° rotation with an acquisition time of 10 s at room temperature (23 ± 0.5°C). For each sample, the spectra were routinely recorded every 5 min for at least 30 min. The photocurrent output from the light detector was digitized and the data were transferred to the hard disk of a computer. Each curve was defined by ≈300 experimental points, all lying within ±1° of the resonance angle.

The SPR spectrum is defined by the optical properties (extinction coefficient, k; refractive index, n) and the thickness of the film t. The addition of a thin layer of a dielectric material such as lipid or protein to the metal surface usually shifts the position of the resonance minimum toward larger incident angles and the SPR curve is broadened. The amplitude of these changes is related to the optical properties and the thickness of the added film, which can be estimated by nonlinear least-squares fitting of the experimental SPR curve to the theoretical reflectance as described (10, 19–22).

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Abbreviations: apoLp-III, apolipophorin III; DG, diacylglycerol; SPR, surface plasmon resonance; PC, phosphatidylcholine.

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The mass of adsorbed protein was estimated from the refractive index of the protein layer and its thickness, by using the Lorentz–Lorenz equation for the refractive index of a mixture of buffer and protein (19, 23).

Lipid Film Formation. Lipid bilayer films were prepared as described (10) with minor modifications. Egg phosphatidylcholine (PC; 8 mg/ml) and *sn*-1,2-diolein (DG; Avanti Polar Lipids) at the desired molar ratios were dissolved in butanol:squalene, 100:3 (vol/vol). The diolein was kept dry at −20°C and fresh solutions of the lipid were prepared just before each experiment. The selfassembled bilayer membrane was prepared by spreading 4 μl of the lipid solution across an orifice in a Teflon sheet (4 mm in diameter) that separates the silver film from the aqueous solution. Further details on membrane formation and structural characterization have been reported (10).

The sample cell (1.5 ml) was filled with 5 mM Na_2HPO_4 (pH 7.0). ApoLp-III was purified essentially as described (24), and the addition of 5 μl of the stock protein solution (0.35 mg/ml) was done with a 10-μl Hamilton microsyringe.

RESULTS

Fig. 1*A* shows the SPR spectra obtained upon successive additions of apoLp-III to an egg PC membrane containing 2 mol % diolein (spectra 2–6), as well as the spectrum of the bare metal. The least-squares fitting procedure for the SPR spectrum of the bare silver film was performed by systematically adjusting the real and imaginary parts of the complex refractive index and the film thickness (t) to minimize the global error between the observed and computed values of the reflectance curves around the resonance minimum (±1°). After the addition of lipid, it was assumed that the parameters previously obtained for the bare metal remained unchanged. Therefore, to achieve a fit to the data for the lipid layer, it was only necessary to vary the thickness and the complex refractive index of the lipid. After the addition of the protein, it was assumed that the parameters of the metal and lipid layers did not change and the data were fit as before. Fig. 1*B* shows experimental spectra 2 and 6 from Fig. 1*A* superimposed on the theoretical curves obtained by the fitting procedure. The iterative fitting process yielded small standard deviations, usually between ±0.01 and ±0.02, indicating good accuracy in the optical parameter estimations (Δn = ±0.01 and Δt = ±1 Å).

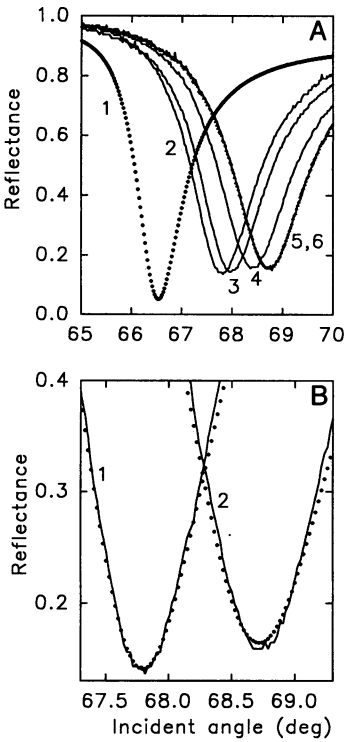

FIG. 1. (*A*) SPR spectra (reflectance vs. incident angle). Spectra: 1, bare silver metal film of 48.6 nm; 2, egg PC/DG (98:2, mol/mol) bilayer in buffer; 3–6, bilayer in trace 2 in the presence of 62 nM, 125 nM, 188 nM, and 250 nM apoLp-III, respectively. In all cases, the binding of apoLp-III to the bilayer was rapid, reaching equilibrium in ≈10 min. (*B*) Experimental reflectance curves of spectra 2 and 6 (solid line) from *A*, labeled traces 1 and 2, respectively, and theoretical reflectance curves obtained upon fitting (dotted line).

All spectra of Fig. 1 and those obtained with membranes containing up to 2.5 mol % DG consist of one resonance that can be fitted to a defined set of optical parameters. The presence of only one plasmon resonance in the SPR spectra indicates the absence of lateral heterogeneity, at least in the macroscopic field defined by the area of the laser spot and the propagation lengths of the plasmon surface waves, which at 633 nm is ≈40 μm (25).

Fig. 2*A* and *B* shows the dependence of the thickness of the protein layer t and the difference in refractive indices of the protein layer and buffer ($n_p - n_b$) on the concentration of apoLp-III for egg PC membranes that differ in their DG content. The small (10%) apparent increase in the calculated thickness as the apoLp-III content increases may reflect a small reorientation of the apoLp-III on the bilayer surface as

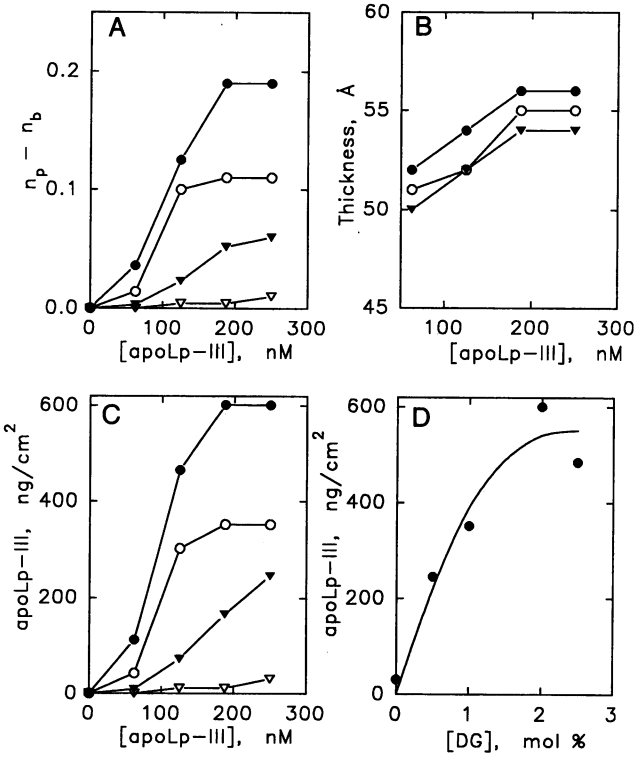

FIG. 2. Dependence of the optical parameters $\Delta n = n_p - n_b$ (*A*) and t (*B*) of the protein layer on the concentration of apoLp-III for the egg PC bilayers containing 0 mol % DG (▽), 0.5 mol % DG (▼), 1 mol % DG (○), or 2 mol % DG (●). n_p, Refractive index of the protein layer of thickness t; n_b, refraction index of the buffer. The parameters were obtained from fitting the resonance curves around the resonance minima. Between 0 and 2.5 mol % DG, the thickness of the lipid bilayers (in the absence of protein) did not change and had an average value of 67 ± 0.5 Å (n = 5). (*C*) Mass of adsorbed protein as a function of the total apoLp-III concentration. Because only a small fraction of the added apoLp-III binds to the lipid film, which has a total area of ≈0.4 cm^2, the concentration of unbound apoLp-III is close to the total concentration of apoLp-III. (*D*) Maximum apoLp-III binding as a function of the concentration of DG in PC membranes.

its packing density increases (see below). Fig. 2*C* shows the binding curves obtained at four DG concentrations. The mass of the protein bound was calculated by using the Lorentz–Lorenz equation for a binary mixture of protein and buffer, a procedure that has been validated (23).

Increasing the concentration of DG in the bilayer does not affect the binding affinity of apoLp-III, which remains almost constant at an average K_d value of 100 nM. Therefore, the increased binding of apoLp-III to membranes containing increasing concentrations of DG is due to an increase in the number of binding sites.

Fig. 2*D* shows the effect of the concentration of DG in the membrane on the binding of apoLp-III at saturating concentrations of apolipoprotein. The plateau in the plot indicates that no further increase in the number of binding sites takes place when the concentration of DG is increased above 2 mol %. From the average values of the refractive index (n = 1.51) and the thickness of the protein layer (t = 55 Å) obtained at 2.0 and 2.5 mol % DG, we conclude that a highly packed protein layer ≈55 Å thick is formed. By using the Lorentz–Lorenz equation, we estimate that apoLp-III occupies 70% of the volume of this 55-Å-thick layer. The maximum mass of protein bound to the membrane, at saturation, is calculated to be ≈600 ng/cm^2 or 33 pmol of apoLp-III per cm^2. From these data, it is calculated that the apoLp-III molecule occupies ≈500 Å^2 at the membrane surface. This molecular area is very close to that determined for apoLp-III at high pressures at the air–water interface (26) and is consistent with a disposition of apoLp-III on the membrane surface in which the long axis of the protein molecule is normal to the membrane surface. This binding configuration is further confirmed by comparing the thickness of the protein layer, 55 Å, with the length of the long axis of the prolate ellipsoid protein determined crystallographically, 56 Å (6). A reasonable value for the molecular area of PC in a bilayer is 60 A^2, and if we consider that saturation of the membrane with apoLp-III requires the presence of 2 mol % DG, we calculate that the presence of one molecule of DG promotes the binding of six molecules of apoLp-III.

Fig. 3 shows the spectra of an egg PC film containing 6 mol % DG in the presence and absence of apoLp-III. Comparison of the spectra of the lipid films containing pure egg PC and egg PC containing 6 mol % DG shows that, at this concentration, DG produces a large angular shift of the reflectance minimum and broadening of the SPR spectrum. The increase in the thickness of the lipid film, from 67 Å at 2.5 mol % DG to ≈110 Å (value obtained from fitting of curve 3) at 6 mol % DG, suggests that a nonbilayer lipid structure is present. The addition of apoLp-III produced even larger spectral changes and a clear lateral heterogeneity. The spectrum shows two well-resolved resonances: the one at the left belongs to the bare metal film, whereas the resonance that appears at ≈74° is due to a large lipid–protein complex with a diameter of ≈500 Å.

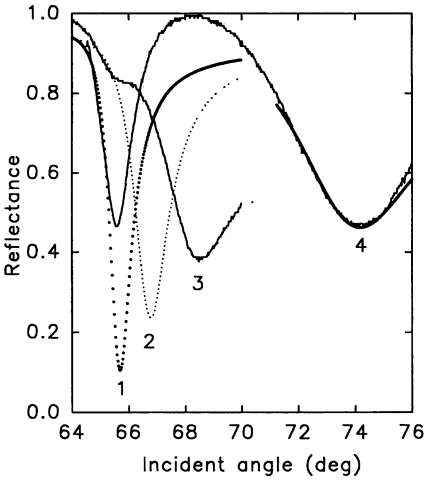

FIG. 3. SPR spectra. Spectra: 1, bare metal film, 46.8 nm; 2, 100% egg PC lipid film; 3, lipid film of egg. PC containing 6 mol % of DG; 4, lipid film as for spectrum 3 in the presence of 125 nM apoLp-III (dotted curves) and the theoretical curve obtained after fitting (solid curve).

The presence of a resonance due to bare metal shows that another type of lipid–protein interaction takes place at a DG concentration of 6 mol %. The formation of larger lipid–protein complexes requires more lipid, which is obtained by stripping the PC molecules from the nascent metal surface. An intermediate situation was observed when the lipid film contained 4 mol % DG. In this case, the lipid film also seemed to contain nonbilayer regions and the addition of apoLp-III, although leading to the formation of a large lipid–protein complex, did not strip the phospholipid from the metal surface (data not shown).

DISCUSSION

Effect of DG on Membrane Properties. Low concentrations of DG have been shown to promote the formation of nonbilayer lipid phases in liposomes (27, 28), membrane fusion (28, 29), and changes in cell morphology (30). The perturbing effect of DG on the packing of phospholipids observed in natural and artificial membranes is believed to be due to the small polar head group of the DG molecule that, when inserted into a bilayer, exposes the hydrocarbon chains of the phospholipids to the aqueous medium (27). As little as 2 mol % DG was shown to decrease the transition temperature for the lamellar/inverted hexagonal phase transition in dioleoylphosphatidyl monomethylethanolamine (28) and egg phosphatidylethanolamine (27), but relatively higher concentrations of DG (≈10 mol %) were necessary to affect the lamellar–hexagonal transition in egg PC bilayers (27). Relatively low concentrations of DG, 4 mol %, promoted the formation of nonbilayer structures in our system, which may reflect the intrinsic instability of planar bilayers containing a single phospholipid in the self-supported membrane employed in this study (31).

At concentrations below those required to promote the formation of nonbilayer structures, DG still seems to produce major changes in the packing of the PC bilayer. Thus, the fact that at a DG concentration of 2.5 mol % or lower, each DG molecule promotes the binding of six apoLp-III molecules would indicate that each DG molecule is affecting an area of ≈3000 Å^2 or the packing of ≈50 molecules of PC.

Role of DG in apoLp-III Binding to Phospholipid Surfaces. Previous *in vitro* studies showed that, in some model systems, apoLp-III binds to PC or other phospholipids (26, 32–34). However, apoLp-III does not bind to lipoprotein particles whose surface appears to be covered almost exclusively by phospholipid (4, 35, 36). The presence of DG on the lipoprotein surface seems to be necessary to trigger the binding of apoLp-III. *In vitro* experiments of DG loading and hydrolysis are also consistent with the requirement of DG for apoLp-III binding (37–39). The low degree of binding of apoLp-III to the egg PC bilayer in the absence of DG and the large increase in apoLp-III binding promoted by low concentrations of DG observed in the present study are consistent with the requirement of DG for apoLp-III binding and are also consistent with the proposed (35) highly destabilizing effect of DG on the lipophorin surface. The fact that the increased binding of apoLp-III is due to a change in the number of binding sites, but not to a change in the dissociation constant, indicates that at low concentration, each DG molecule produces local noninteractive perturbations in the phospholipid packing.

Mechanisms of the Interaction of apoLp-III with Phospholipid Surfaces. The molecular structure of apoLp-III has been

determined at 2.5 Å (6). The protein is composed of five long amphipathic α-helices connected by short loops. The hydrophilic faces of the helices are pointing toward the aqueous face and the hydrophobic residues are pointing into the interior of the protein. A similar arrangement was subsequently determined in the crystal structure of the N-terminal domain of human apolipoprotein E (40). To date, apoLp-III molecules from four insect species have been sequenced (41). All apoLp-III molecules seem to contain five amphipathic α-helices of similar characteristics and two conserved leucines that form part of the hydrophobic loops located between adjacent amphipathic helices 1 and 2 and helices 3 and 4.

A study of the molecular area of apoLp-III at the water–air interface indicated that apoLp-III can adopt two conformations. In one that is observed at low surface pressures, apoLp-III forms a monolayer in which each molecule occupies 4000 A^2. The second conformation is observed at high surface pressures where apoLp-III occupies 480 A^2 per molecule (26). The latter study combined with the crystal structure of apoLp-III gave the basis for a model of apoLp-III binding to lipid surfaces (6). This model suggests that apoLp-III binds to DG in the surface of lipophorin via the hydrophobic loops, which are both located at one end of the long axis of the prolate ellipsoid-like molecule. After the initial binding, it was proposed that apoLp-III would undergo a conformational change that would allow the interaction of the hydrophobic side chains of the α-helices with the lipid (6). The results obtained in the present study give experimental support to this two-step binding model and allow us to rationalize further the nature of the apoLp-III–lipid interaction.

First Binding Step. In the DG concentration range from 0 to 2.5 mol %, the thickness of the protein layer and the mass of protein bound to the lipid surface are consistent with an interaction in which apoLp-III lies with its long axis normal to the bilayer surface and does not significantly disrupt the lipid structure. In the concentration range from 0 to 2.5 mol %, each DG molecule represents an independent perturbation of the membrane structure, a defect that is recognized by apoLp-III. The fact that in the low DG concentration range there is no change in the binding constant further confirms that between 0 and 2.5 mol % DG, the same type of lipid–protein interaction is taking place. The hydrophobic loops, which contain the conserved leucines, might constitute the sensor that allows the detection of hydrophobic spots (defects) in the surface of membranes or lipoproteins and provides a primary site for binding of apoLp-III to the surface. In fact, our results indicate that apoLp-III recognizes and binds to a PC membrane containing as little as one DG molecule. This primary association, although of high affinity, does not include a major conformational change in apoLp-III, which would be bound to the lipid surface through the interaction of only a few amino acid residues. Although triggered by the presence of small defects, the binding of apoLp-III is expected to involve not only van der Waals interactions but also electrostatic and H-bond interactions with the phospholipid polar head groups. Fig. 4 shows a schematic representation of the binding of apoLp-III, at saturating concentrations of protein, to a phospholipid bilayer containing defects induced by DG.

Second Binding Step. ApoLp-III contains class A amphipathic α-helices (7, 41) that are characterized by a high density of positively charged residues along the sides that separate the hydrophobic and hydrophilic faces of each helix. These kinds of α-helices are believed to interact strongly through both electrostatic interactions with the negatively charged phosphate groups of the phospholipid, and hydrophobic interactions with the hydrocarbon chains of the phospholipid molecules (7, 42). The formation of large lipid–protein complexes and the destruction of the phospholipid bilayer that takes place upon the interaction of apoLp-III with PC membranes containing >4 mol % DG are the result of a stronger lipid–protein interaction than that which occurs in the first binding step. When the membrane contains 4 mol % DG, or more, the interaction of apoLp-III with the membrane cannot be described by a single high-affinity adsorption process. At >2.5 mol % DG, the dimensions of the lipid film are consistent with the presence of nonbilayer structures, which indicates that the perturbation generated by each DG molecule cannot be represented by small noninteracting defects. Thus, at >2.5 mol %

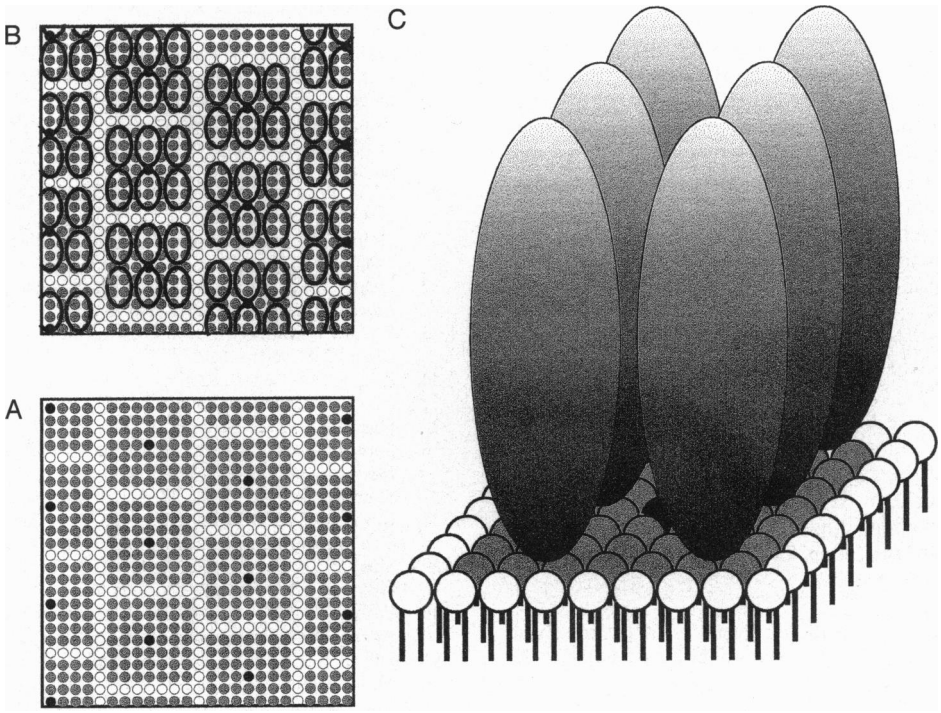

FIG. 4. Schematic representation (viewed from above). (*A*) The production of isolated defects in the phospholipid surface (shaded circles) by DG molecules (solid circles). (*B*) The binding of apoLp-III (ovals), at saturating levels of protein, to these defects. (*C*) Enlarged view of the binding of six apoLp-III molecules (shaded ovals) to the defect caused by one DG molecule at saturating concentrations of the protein.

DG, the formation of large DG-rich clusters could accommodate a major conformational change in apoLp-III, allowing a new type of lipid–protein interaction. This interaction would resemble the typical interaction that takes place when apolipoproteins are incubated with liposomes at the transition temperature of the phospholipid. The large number of defects found at the boundaries of the coexisting liquid–crystalline and gel phases promotes the formation of disc-like structures that involve a change in both the lipid structure and the conformation of the protein. These "large defects" would have sizes comparable to the lengths of the amphipathic α-helices and, therefore, could accommodate and stabilize a major conformational change in the apolipoprotein.

Why Does apoLp-III Have the Ability to Bind to a Lipid Surface in Two Ways? We think that there are two answers to this question: the first relates to the stability requirements of apoLp-III and the second relates to the properties and/or stability of the lipoprotein. (*i*) The ability of apoLp-III to bind to a hydrophobic spot on a phospholipid surface by means of the small hydrophobic site contained in the loops at the end of the molecule is necessary because, otherwise, to interact hydrophobically with the lipid surface, the apolipoprotein, which has the hydrophobic sides of the amphipathic α-helices buried in the interior of the protein, would have to unfold in solution before reaching the lipid surface. The exposure of the hydrophobic faces of the amphipathic α-helices to the aqueous medium would trigger aggregation of the protein. Thus, the presence of the small hydrophobic loops guarantees the localization of apoLp-III to the surface defect, and if that hydrophobic spot grows large enough, the apolipoprotein could change its conformation and bind through the α-helices. (*ii*) A small defect on the lipophorin–lipid surface, as is observed with <4 mol % DG, would not be large enough to allow the binding of apoLp-III through its α-helices but could be large enough to promote the aggregation of the lipoprotein particles. Thus, the ability of apoLp-III to bind, through its sensing loops, to small lipid defects in the lipoprotein surface could also be a way of preventing the aggregation and precipitation of lipoprotein particles containing small amounts of DG in their lipid surface. Such defects could be readily produced during the metabolism of the insect lipoproteins.

Concluding Remarks. In this report we present strong evidence supporting the ability of apoLp-III to bind to a phospholipid surface with its long molecular axis normal to the lipid surface. The experimental demonstration of a primary binding step, or recognition step, in the association of a typical exchangeable apolipoprotein with a lipid surface and the rationale for its existence leads us to suggest that this could be a general binding mechanism for such apolipoproteins. The localization of the sensing domain might vary with the type of apolipoprotein. It should be possible, by using site-directed mutagenesis and SPR studies with supported lipid bilayers, to identify such binding domains.

Furthermore, as far as we know, the strong effect of small concentrations of DG on the binding of a protein to a membrane reported here has not been observed, or reported to take place, in any other model system. Perhaps the self-assembled supported membrane system not only is a system that allows one to perform SPR and electrochemical studies but also is a system that might allow the observation of physiologically relevant processes that are not seen when using other model membrane systems.

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