brief communication

Two-dimensional recognition pattern of lipid-anchored fab' fragments

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ABSTRACT A two-dimensional pattern of oriented antibody fragments was formed at the air–water interface and transferred onto a solid support. The Fab'-fragments of a monoclonal antibody against the hapten dinitrophenyl (DNP) were covalently linked via a hydrophilic spacer to phospholipid vesicles. A monomolecular lipid–protein layer at equilibrium with these vesicles was allowed to form at the air–water interface. The monolayer was separated from the vesicle phase and transferred to a Langmuir–Blodgett trough. By cooling and compressing, the previously homogeneous lipid–protein film was driven into a two-dimensional phase separation resulting in protein-rich domains and a second phase consisting mainly of lipid. This film was transferred onto a solid support in a way that preserved the protein–lipid pattern. The specificity as well as the contrast in the binding activity of the two different separated phases were then quantified using microfluorometry. DNP conjugated to fluorescein-labeled bovine serum albumin (BSA) showed virtually no binding to the lipid regions, but gave a ratio of bound DNP-BSA to Fab'-lipid of >50% in the protein-rich domains proving that the Fab'-moiety retained its biological activity. This demonstrates that the technique presented here is well suited to modify different solid surfaces with a pattern of a given biological function. The optional control of lateral packing and orientation of the components in the monolayer makes it a general tool for the reconstitution of supported lipid–protein membranes and might also open new ways for the two-dimensional crystallization of proteins at membranes.

INTRODUCTION

Considerable interest has developed in recent years in the modification of solid surfaces with functional groups like proteins or polysugars. Methods that allow the mimicry of cell surfaces on an underlying solid support have drawn particular attention. This effort is stimulated by potential applications in the field of biosensors as well as by the use of surface sensitive techniques to monitor events on cell membranes or equivalent model systems (1, 2). In general it is an advantage to design a system in a way such that it contains its intrinsic control. This can be realized best by a pattern of active and inert or neutral sites which are separated into two phases preserved on the same support. A recent article (3) emphasizes a high-affinity binding system at the air–water interface, using streptavidin and various biotin lipids. As a functional group we used the Fab'-fragment of a murine monoclonal antidinitrophenyl (DNP) antibody (4). The Fab'-fragment was covalently linked to a phospholipid (see Fig. 1) to create a membrane bound model receptor (5; Egger, M., S. P. Heyn, and H. E. Gaub. Manuscript submitted for publication.). The choice of a monoclonal antibody's binding site as receptor has the obvious advantage that the system developed is very general and may be applied to a wide range of problems. The way our model receptor is designed is in very close analogy to the molecular schematics of the growing family of lipid coupled membrane bound receptors (6). We believe that Fab'-lipids could potentially mimic some general features of these and other surface molecules which in most cases are very difficult to isolate.

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MONOLAYER FROM VESICLES

Two principal techniques have been established which allow the formation of lipid bilayers containing proteins on solid supports. The first technique is based on the surface-induced fusion of vesicles resulting in a bilayer on the surface (7). This technique is very simple to apply and incorporated proteins have been shown to maintain their integrity and function, but the lack of control of the thermodynamic parameters limits its applications. The other widely used technique, the Langmuir–Blodgett technique, generates supported lipid layers by the multiple transfer of a monolayer at the air–water interface onto the substrate (8). This method allows the control of pressure, area, temperature, and to a certain extent the lateral distribution of the components. Problems arise, however, when attempts are made to incorporate proteins into the monolayer as it is usually spread from an organic solvent, by allowing the solvent to evaporate at the surface. It was reported some years ago that under certain conditions the lipid in a vesicle solution is in equilibrium with the air–water interface resulting in a lipid monolayer (9, 10). The kinetics of the lipid exchange between bulk and surface is a sensitive function of the state of the lipid. Below the liquid–solid phase transition temperature of the

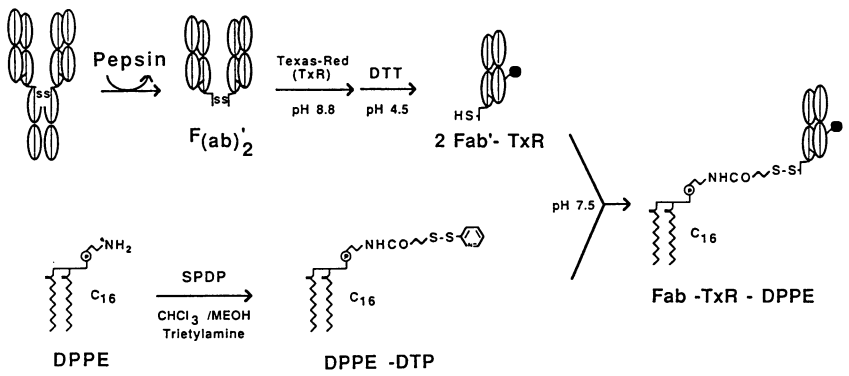

FIGURE 1 Synthesis of the fab'-lipid. Anti-DNP monoclonal antibodies derive from the cell line ANO2 (2). Hybridoma cells were grown in culture medium RPMI 1640, 1 mM pyruvate, 1 mM glutamate, supplemented with 1% fetal calf serum (Seromed, Berlin, FRG). The affinity-purified antibodies were cleaved with pepsin. This yields bivalent $F(ab')_2$-fragments with intact disulfide bonds at the hinge region to which a spacer lipid is covalently coupled. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Before cross-linking, the $F(ab')_2$-fragments were labeled with Sulphorhodamine 101 (Texas Red; Molecular Probes, Eugene, OR) and reduced to monovalent Fab'-fragments. 50 mM Dithiotreitol at pH 4.5 was used to keep the intrachain and heavy/light-chain disulfide bonds intact. After the procedure developed in (5), in parallel, the reactive phospholipid dipalmitoyl L-α-phosphatidyl-ethanolamine (DPPE) was coupled to a heterobifunctional cross-linker, here *N*-succidimidyl 3-(2-pyridyldithio) propionate (SPDP; Pharmacia, Freiburg, FRG). The purified spacer lipid dipalmitoyl-L-α-phosphatidylethanolamine 3-(2-pyridyldithio) propionate (DPPE-DTP) can be stored under argon for several months without significant decay. The coupling reaction between spacerlipid and labeled antibody fragment was carried out in 0.01 M Hepes 150 mM NaCl, pH 7.45. As the spacerlipid does not form stable vesicles it is mixed with dimyristoyl L-α-phosphatidylcholine (DMPC). Small unilammellar vesicles were prepared by sonication for 10 min in coupling buffer using a tip sonifier (Branson, IL). After adding Fab'-fragments to such a vesicle solution the mixture is kept at 25°C for 16 h. A molar ratio of 0.04 of DPPE-DTP to DMPC and a final concentration of protein of 1.2 mg/ml yields the highest amount of Fab'-lipid; further details are given by Egger, M., S. P. Heyn, and H. E. Gaub (manuscript submitted for publication). The resulting Fab'-vesicles are finally purified either by gel filtration on Sepharose CL 4B or by density gradient centrifugation in a metrizamid (Serva, Heidelberg, FRG) gradient.

vesicles the exchange is negligible in the time scale of minutes. We used this approach to form a lipid monolayer containing our model receptor from the vesicle solution. The monolayer is allowed to form above the main transition temperature. Due to the lateral pressure gradient the monolayer expands and moves over a buffer bridge onto the surface of a Langmuir-Blodgett trough stripping off the vesicles which were at the surface. A so obtained lipid monolayer exhibits essentially the same pressure area diagram as a monolayer of pure lipid obtained by spreading from an organic solvent (Heyn, S. P., M. Egger, and H. E. Gaub. Manuscript submitted for publication.).

PHASE SEPARATION

In recent years several groups have studied the formation of pattern in pure lipid monolayers at the air–water interface (11, 12). In most cases the observed patterns were formed by the phase separation in the liquid–solid coexistence region. To a certain extent size, geometry, orientation, and density of the solid domains can be controlled by experimentally accessible parameters and predicted from theoretical considerations (13). We have used this approach to generate a two-dimensional pattern of our model receptor. A lipid-protein monolayer at the air–water interface was allowed to form following the procedure given in Fig. 2. At low pressures and high temperatures the Fab'-lipid and the filling lipid dimyristoyl L-α-phosphatidylcholine form a homogeneous two-dimensional fluid. At pressures >30 mN/m a phase separation into a protein-rich phase and an essentially pure lipid phase occurs. This situation is shown in Fig. 3, where the protein was fluorescent labeled with Texas Red and thus appears bright against the dark background of the unstained lipid. The resulting patterns, where dark domains in bright environment and vice versa coexist, suggest that both phases have the same symmetry. In the one case the pure lipid phase and in the other case the protein rich phase is continuously connected. Observation of the fusion of single bright domains as well as of single dark domains indicate that both phases are fluid. Due to the fusion process the average domain size increases in time and reaches some 10 μm within minutes after the phase separation occurred. This type of transition has not yet been reported for a pure lipid monolayer under comparable conditions. We therefore interpret this phase separation as being dominated by the protein part of the molecule. In this model the major function of the lipid part would be to confine the hybrid molecule to the air–water interface and to determine its orientation with respect to this plane.

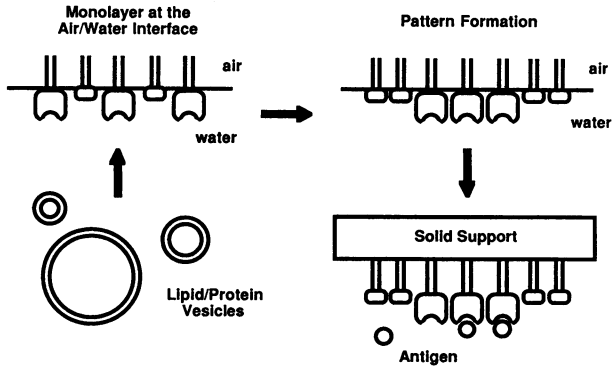

FIGURE 2 Formation of a lipid–protein monolayer at the air–water interface from a vesicle solution, transfer to the Langmuir–Blodgett trough and coating onto the solid support. The experimental setup consists of a miniature Langmuir-Blodgett trough system (details are given by S.-P. Heyn, M. Egger, and H. E. Gaub [manuscript submitted for publication]) and a temperature controlled well where vesicles spread a monomolecular film at the air–water interface. A thin film of buffer on a strip of filter paper bridges the two compartments and allows the lipid–protein monolayer to expand onto the surface of the Langmuir trough subphase while the vesicles are retained in the spreading well. A fluorescence microscope on an xy-stage allows the determination of the lateral distribution of fluorescent markers in the monolayer. Recordings are made using a SIT camera interfaced to an image analyzing system. The local fluorescence intensity at the emission wavelength of the used fluorescent labels is measured from a 20-μm spot using an attached photomultiplier. The monolayer is transferred onto a solid support by the horizontal dipping technique where the solid support is either precoated with a lipid monolayer (resulting in a supported lipid bilayer) or with covalently linked hydrocarbon chains. The sample ends up in a chamber which can be removed from the trough for further experiments. The system is kept in a humid atmosphere.

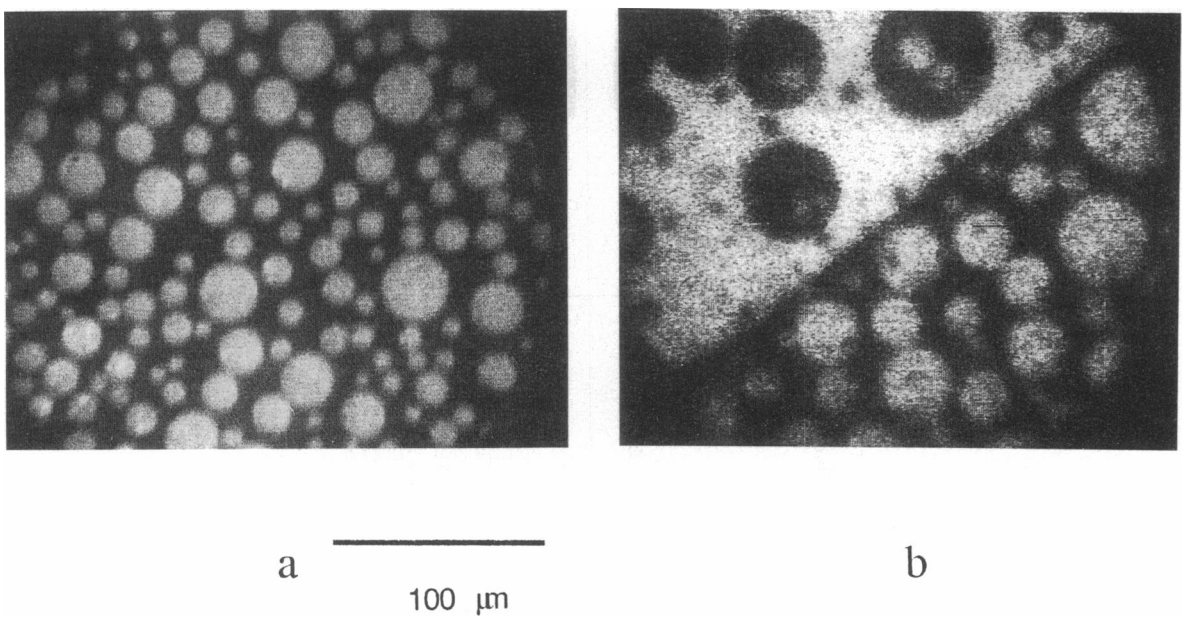

FIGURE 3 Fluorescence micrographs of Fab'-lipid monolayer at the air–water interface. Bright domains indicate Fab'-lipid which is labeled with Texas Red fluorescent dye (λex = 580 nm, λem = 620 nm). (*a*) The image was taken 2 min after the phase separation occurred at a lateral pressure of π = 33 mN/m and a temperature of υ = 18°C. (*b*) Same film and conditions as in (*a*) but 10 min after the start of the phase separation. The monolayer was obtained as follows: A vesicle suspension prepared as described in Fig. 1 was applied to the spreading well. The temperature there was 38°C whereas the Langmuir trough was kept at 18°C. The surface pressure in the Langmuir trough was measured and the wet bridge was removed when the surface pressure reached 15 mN/m indicating the transfer of a sufficient amount of lipid–protein. Before transfer the Langmuir trough surface was aspirated several times to remove remaining surface active impurities. After the transfer, the fluorescence of the Fab'-lipid monolayer appeared uniform and exclusively at the surface. The film was then compressed to a final pressure of 38 mN/m. At pressure values of 33 mN/m a lateral phase separation occurred and fluorescent and nonfluorescent domains appeared.

TRANSFER ONTO SOLID SUPPORT

When transferred onto a solid support the lateral mobility of the lipid in a monolayer depends strongly upon the surface properties of the solid support (14). We have covalently coupled alkyl chains 18 C atoms long onto coverglasses and used these as supports for the lipid–protein monolayers, resulting in a virtually complete immobilization of the lipids and proteins after the transfer. This surface treatment has been shown to be applicable to a broad variety of substrates (15) making this procedure relevant to a wide range of systems. When kept submerged, this structure proved to be stable for at least several days. The result is shown in Fig. 4 *a*. The domains are well preserved. Control measurements with surface plasmon microscopy (16) revealed that within the lateral resolution of 2 μm the thickness of the protein-rich phase was constant and gave a height step of 40 Å on top of the filling lipid.

TWO-DIMENSIONAL RECOGNITION PATTERN (FLUORESCENCE BINDING ASSAY)

The supported sample was incubated with bovine serum albumin (BSA) carrying an average of five DNP haptens

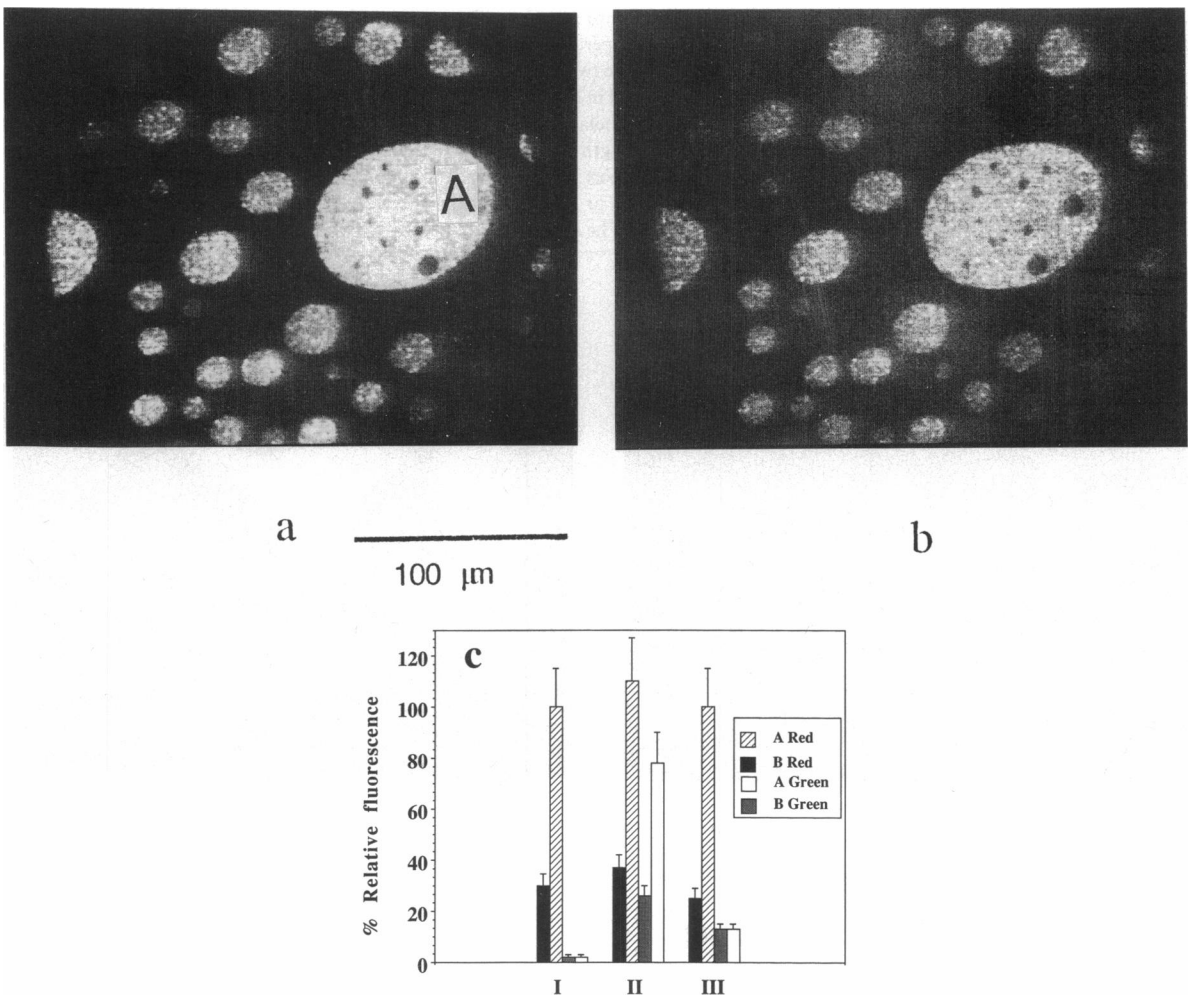

FIGURE 4 Binding assay of transferred Fab'-lipid pattern. (*a*) Texas Red fluorescence of the Fab'-lipid after transfer onto a cover glass which was alkylated with octadecyltrichlorosilane (Petrarch Systems Inc., PA) following the procedure of reference 11. (*b*) FITC fluorescence (λex = 490 nm, λem = 530 nm) of the same spot after incubation for 20 min at 37°C with fluorescein labeled DNP-albumin (F-DNP-BSA). The concentration of F-DNP-BSA was 30 nM in 0.01 M Hepes buffer 150 mM NaCl pH 7.4 containing a 10-fold excess of unlabeled albumin. The chamber was washed with the at least 20-fold volume to remove unbound antigen. (*c*) quantitative analysis of the fluorescence intensity measured from a 20-μm spot in the Fab'-lipid domain (*A*) and in the filling lipid regions (*B*) before (first group) and after addition of F-DNP-BSA (second group). As negative control (third group) fluoresceineted BSA carrying no DNP-hapten was used. The fluorescence was averaged over 20 spots in 3 samples and normalized by the number of labels per molecule where the Texas Red signal of the protein rich region was set to 100%.

and eight fluorescein groups per BSA molecule in a 10-fold excess of unaltered BSA. After washing, the sample showed a clear congruence between red and green fluorescence (Fig. 4 *b*). The contrast between the protein rich domains and the lipid background observed by green fluorescence was only slightly smaller than that observed by red fluorescence. Another protein carrier, labeled Ovalbumin, was used resulting in comparable binding characteristics (data not shown). As a negative control fluorescein-labeled BSA lacking DNP groups was applied in the same procedure and resulted in virtually no contrast in the green fluorescence (see Fig. 4 *c*). To quantify the binding and to circumvent possible interference caused by the different spectral properties of the different dyes a separate binding experiment was carried out. Here Texas Red as label on both antigen and Fab'-lipid was used. In this case the measured fluorescence ratio with and without antigen was, with the known fluoresceination, directly convertible to a ratio of bound antigen to binding site. We obtained a ratio of 1:1.9, which means that at least every second antibody fragment is capable of binding its antigen.

CONCLUSION

These results show that (*a*) the majority if not all of the binding sites are accessible to the DNP-carrier which means that the Fab'-moiety is arranged in the two-dimensional pattern in a predominantly oriented way; (*b*) the antigenic determinant is recognized and selectively bound by the functional groups which means that the Fab'-part of the molecules has retained its activity on the solid surface; and (*c*) the lipid membrane effectively protects the solid surface from nonspecific adsorption giving rise to a high contrast between bound and free (control) areas on the same surface.

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