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Synthetic lipid-anchored receptors based on the binding site of a monoclonal antibody

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Highly specific ligand receptor interactions generally characterize molecular recognition at cell surfaces and other biological systems. In this study we simulate a membrane receptor by fusing a monoclonal antibody fragment to a phospholipid. A sulfhydryl group in the hinge region of a monoclonal antibody fragment, was covalently linked to derivatives of phosphatidylethanolamines and phosphatidylserine via three different hydrophilic spacer arms. We investigated and characterized these lipid-anchored Fab-fragments which we have named 'Fab-lipids' in liposomal and monolayer systems. Methods for the monomolecular assembling of such films at the air/water interface and techniques used for their manipulation are outlined. We describe two possibilities for building a monomolecular receptor layer, consisting of two-dimensional pattern of oriented Fab-fragments with their artificial hydrophobic anchor embedded in a lipid matrix. In the first method a monomolecular film at the air/water interface was allowed to form from a vesicular suspension and driven into a phase separation, resulting in protein rich domains embedded in a protein depleted phase. This film was transferred onto a solid support in such a way that the established pattern was preserved. Alternatively, a recognition pattern was formed by directly cross-linking the Fab-fragments to preformed planar membranes composed of the reactive spacer-lipids and an inert matrix lipid. Specificity as well as contrast of the binding activity of the receptor layers were quantified using micro-fluorimetry.

Introduction

Membrane attachment of eukaryotic proteins via hydrocarbon anchors is attracting considerable interest [1,2]. An increasing number of physiologically important proteins is known to contain a hydrophobic anchor, either fatty acids (i.e. myristic, palmitic acids) or complex glycophospholipids (glcosylphosphatidylinositol) [3,4]. The functional role of this modification aside from membrane attachment and dynamics is still unclear and whether putative recognition processes at cell membranes are involved remains to be revealed [5].

In order to study this type of questions we designed a model system from a univalent binding site af a monoclonal antibody to which a variety of lipids were attached (Fab-lipids). The approach presented here has the major advantage that with the help of the monoclonal antibody technology we were supplied with

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Abbreviations: DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (di-16:0); DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine (di-18:0); DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylserine (di-14:0); DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (di-14:0); PDP-PE, propionyldithiopyridonephosphatidylethanolamine; MPB-PE, maleimidophenylbutyrylphosphatidylethanolamine; MB-PE, maleimidobenzoylphosphatidylethanolamine; NBD-PC, 1-palmitoyl-2-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanyl]phosphatidylcholine; SPDP, *N*-succinimidyl 3-[2-pyridyldithio]propionate; SMPB, succinimidyl 4-[*p*-maleimidophenyl]butyrate; MBS, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl; TxR, Texas red (Sulforhodamine 101); FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; OA, ovalbumin; DNP, 2,4-dinitrophenol-hapten; TLC, thin-layer chromatography; Fab, antigen binding fragment.

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an abundant amount of a membrane attached protein well characterized in its structure and function.

The synthetic procedures described result in an easily reducible disulfide bond using SPDP crosslinking reagent or lead to a stable thioether linkage with maleimides of different spacer length (SMPB and MBS). In each case one of the thiol-groups at the hinge region of a freshly reduced monoclonal F(ab')$_2$ fragment is site-specifically coupled to the derivatized lipid headgroup, thus assuring that the binding site is freely accessible for the antigen. Alternatively carboacyl-derivatized lipids were linked to aminogroups of the Fab fragment, which were not localized. All products were purified from unlinked material through centrifugation on a density gradient. Synthetic procedures similar to the one described in this paper have been used previously for conjugating fatty acids or phospholipids to various proteins [6,7] or peptides [8] and other molecules [9].

In the past protein conjugated liposomes have already been used for various immunochemical and diagnostic purposes, for instance binding of labelled liposomes to cells and the agglutination of cells or latex particles [10]. Specific antibodies attached to the surface of drug containing liposomes enables them to bind to chosen cell surface molecules and to subsequently be internalized by the cells (drug delivery systems) [11].

For certain purposes however, the use of a planar artificial membrane system on solid support offers significant advantages compared to suspended bilayers. Planar membranes doped with natives ligands are accepted by natural cells [12]. As surface sensitive techniques distinguish extremely selective between events close to the surface and those occurring in the bulk phase, such techniques may then be employed to investigate the underlaying molecular interactions.

As depicted in Fig. 1 planar membranes on a solid support composed of lipid-anchored proteins together with a filling lipid can be obtained in three different ways. Vesicle fusion was reported earlier by Brian et al. [13]. We developed two further protocols through which the lateral organization can be controlled by thermodynamic parameters (i.e. lateral pressure, temperature, etc.). Our manipulations resulted in a two-dimensional phase separation of domains rich in Fab-lipid embedded in an essentially pure lipid phase. We have named this contrast image a two-dimensional recognition pattern [14].The use of such 2-dimensional recognition pattern can be manifold: Universal high affinity receptors whose properties can be designed on request might represent a general tool for two-dimensional crystallization. The internal contrast of such recognition pattern and the retained activity makes them attractive for use as biosensing devices, which depend on well defined insulating layers with specific binding activities [15].

Materials and Methods

Reagents. The phospholipids dimyristoyl-DL-α-phosphatidylcholine (DL-α-DMPC 99 + %), dipalmitoyl-L-α-phosphatidylethanolamine (L-α-DPPE 99 + %), distearoyl-L-α-phosphatidylethanolamine (L-α-DSPE 99 + %), bovine serum albumin (BSA), albumin from chicken egg (OA), each essentially free of immunoglobulins and dithiotreitol (DTT) were from Sigma Chem. Co. (St. Louis, MO, USA). Dimyristoylphosphatidylserine (DMPS 99%) and the fluorescent probe 1-palmitoyl-2-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanyl] phosphatidylcholine (NBD-PC) was obtained from Avanti Polar Lipids (Birmingham, USA). All lipids were analyzed by thin-layer chromatography. The heterobifunctional crosslinkers *N*-succinimidyl 3-[2-pyridyldithio]propionate (SPDP), *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) and succinimidyl 4-[*p*-maleimidophenyl]butyrate (SMPB) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) were purchased from Pharmacia/LKB (Freiburg, FRG) and Pierce Chem. Co (Rockford, USA). Octadecyl-

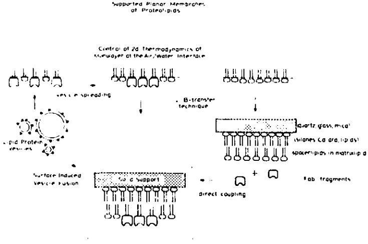

Fig. 1. Schematics showing different methods for the formation of planar supported membranes.

trichlorosilane (OTS) and the haptens 5-fluoro-2,4-dinitrophenol (DNP) and 2,4-dinitrophenol sulfonate were from Sigma and Aldrich (Steinheim, FRG), respectively. Fluorescent dyes Texas red (TxR) and fluorescein isothiocyanate (FITC) were all from Molecular Probes Chem. Co (Eugene, USA). RPMI 1640, pyruvate, glutamate and penicillin/streptomycin were from Biochrom (Berlin, FRG). Gibco BRL (Eggenstein, FRG) supplied us with fetal calf serum (FCS). Metrizamide (99%) was from Serva (Heidelberg, FRG). Supports for column chromatography were all from Pharmacia/ LKB (Freiburg, FRG). Deionized water (Milli-Q-System Millipore Corp., El Paso, USA) was sterile filtered and used throughout the experiments. 10 mM Hepes buffer (Serva, Heidelberg, FRG) pH 7.4 with varying NaCl contents was applied in all experiments unless otherwise indicated. All organic solvents were obtained from Riedl deHaen (Hamburg, FRG) and were of spectroscopic grade.

Monoclonal Fab fragments. The monoclonal antibodies against the dinitrophenol hapten was derived from the cell line AN02 [16] and exhibited a binding constant of $4.5 \cdot 10^6$ M^{-1} for DNP-glycine [17]. Hybridomas were grown in RPMI 1640 medium supplemented with 1 mM pyruvate, 2 mM glutamate and adapted to growth in 1% fetal calf serum. The cells were re-cloned every third month in order to maintain a high rate of antibody production. Isolation of the antibodies from cell culture supernatents was carried out by ammonium sulfate precipitation (35%, 4°C, overnight) followed by affinity chromatography on Protein A-Sepharose 4B. This procedure typically yielded 15–20 mg/l supernatant. Proteolytic cleavage of the whole antibody with pepsin at pH 3.8 and purification by gel filtration (Sephadex G-100) followed by affinity chromatography on a self prepared DNP-lysine agarose support obtained essentially pure $F(ab')_2$ fragments. They were subsequently dialysed and concentrated by ultrafiltration in collodion bags (Schleicher/Schüll, Dassel, FRG). Homogeneity and binding capacity were assured by SDS-PAGE electrophoresis on 10% slab gels and enzyme immunosorbent assay (EIA).

Hapten and fluorochrome conjugation. BSA or OA coupled with the hapten 2,4-DNP were used as antigens in all binding assays with the chosen monoclonal antibody. The coupling procedure is adapted from Balakrishnan et al. [16]. In our case it yielded hapten to carrier ratios of typically 5–8. For the use of fluorescence detection in binding assays, the purified haptenated albumins are optionally reacted with the fluorochromes fluorescein isothiocyanate (FITC) [18] or Texas red (TxR) [19]. The molar ratio of hapten and/or fluorophore to carrier protein is determined by absorption measurements according to the same references. In order to avoid aggregation, the conjugates are kept in Hepes buffer pH 7.0 (no salt added) and centrifuged prior to use. The same procedure was carried out with monoclonal antibody fragments to obtain receptor molecules with a different fluorophore. $F(ab')_2$-fragments were conjugated with the fluorescent dye Texas red, following the procedure of Titus et al. [19]. A method for the estimation of the fluorochrome per protein ratio (F/P ratio) is given in the same reference. Briefly a 10 mg/ml $F(ab')_2$ solution in 0.1 M borate buffer pH 9.0 was mixed with 1 mg TxR, dissolved in anhydrous dimethylformamide. The mixture was kept at room temperature with continuous stirring for 90 min. Free dye was removed from the blue conjugate by passage through a Sephadex G-25 column (30 × 2 cm) equilibrated in 0.01 M phosphate buffer, 150 mM NaCl and dialysis in the dark for 24 h (4°C) against the same buffer containing 0.02% NaN_3.

Synthesis of phospholipid derivatives. As indicated in Table I, propionyldithiopyridone (PDP) derivatives of the two phospholipids DPPE and DMPS were generated by reaction with SPDP. A method first described for phosphatidylethanolamines by Martin et al. [20] was also applied to DMPS with the negatively charged phosphatidylserine headgroup. The modifications are briefly: DMPS (15 µmol) dissolved in 4 ml chloroform/methanol (2:1, v/v) containing 44 mmol freshly destilled triethylamine was added dropwise to a stirred solution of SPDP (25 µmol in 0.5 ml anhydrous methanol). The mixture was incubated for 5 h at room

TABLE I

Coupling efficiencies of the various spacer-lipids

	Spacer-lipid	1 Fab'/lipid (tot.) (µg/µmol)	2 N Fab'/N lipid (µmol 10^{-3}/µmol)	3 N Fab'/vesicles	4 non-specific binding (µg Fab'/µmol lipid)	5 yield (%)
A	PDP-PS	158	3.2	126	< 1	25.3
B	PDP-PE	174	3.5	140	< 1	27.8
C	MPB-PE	308	6.2	246	< 1	49.3
D	MB-PE	89	1.8	71	< 1	14.2
E	HOOC-C12-PE [a]	401	8.0	321	29	59.4 [b]

[a] Data refer to Fab' fragments only.

[b] Yield has not been corrected for non-specific adsorbtion.

temperature under an argon atmosphere and subsequently taken to dryness by rotary evaporation under reduced pressure at 40°C. The residues were redissolved in a small volume of chloroform/methanol (2 : 1, v/v), extracted twice with 0.1 M phosphate buffer, 100 mM NaCl (pH 7.5) to remove water soluble byproducts. The organic phase was then subjected to preparative thin-layer chromatography on PSC Silica gel 60 F254 S plates (Merck, Darmstadt, FRG) to obtain essentially pure PDP-PE or PDP-PS (solvent system: chloroform/methanol/glacial acetic acid/water (60 : 50 : 1 : 4, v/v)). The band which contained the phosphatidylethanolamine or serine derivatives, as indicated by ninhydrin and sulfhydryl negative (Ellmans reagent) and phosphate positive (molybdate spray reagent) spots, was scraped off the plate, dissolved with chloroform/methanol (9 : 1, v/v) and passed through a small column packed with Silica gel 60. The eluate was evaporated to dryness and redissolved in chloroform/methanol (3 : 1, v/v) to a final concentration of 10 mM.

The synthesis and purification of *N*-[4-maleimido] derivatives of phosphatidylethanolamines was accomplished using the same procedure as for the SPDP crosslinker already described. The *N*-hydroxysuccinimide moiety of MBS and SMPB reacts analogously with free deprotonated amino groups. The initial concentration of DPPE was 10 μM and a 1.5-fold molar excess of MBS or SMPB was used. If traces of water were excluded, quantitative conversion of the phosphatidylethanolamines was achieved, as indicated by TLC. Before preparative thin-layer chromatography the chloroform phase was in these cases first extracted one time with 0.1 M phosphate buffer, 100 mM NaCl (pH 7.5) and twice with double-destilled water. After purification the modified phospholipids are stable in chloroform for more than 6 months, when stored at −80°C in glass ampoules under argon. Carboacyl-derivatives of DPPE with a C_{14}-spacerarm were synthesized following a procedure developed by Kung [21]. 1,14-Tetradecanedicarbonic acid was used in all cases for attaching spacer-molecules with a high crosslinking efficiency to the phospholipids.

Liposome preparation. Small unilammellar liposomes (diameter: 30–40 nm) were generated by sonication (tip sonifier, Branson, USA) from lipid suspensions, containing the synthetic reactive spacer-lipid and the matrix lipid DMPC in Hepes buffer. The pure spacer-lipids do not form stable vesicles. Therefore they were mixed with DMPC in a molar ratio of 1 : 20. Chloroform and methanol were evaporated under a N_2 stream and completely removed from the mixture by lyophylisation. The dried film was dispersed on a lab shaker in Hepes buffer and subsequently sonicated for 10 min at 40°C (which is well above the main transition temperature of the matrix lipid) to obtain a 3 mM suspension (total lipid). Following a short relaxation period of 3 h the liposomes were used for the conjugation.

Coupling of Fab-fragments to phospholipid derivatives. The procedures used to prepare Fab-lipids were equivalent in all of the primary coupling and purification steps, except for the pH value in the crosslinking step. As depicted in Fig. 2 the maleimide conjugation results in a stable thioether bond between Fab and the

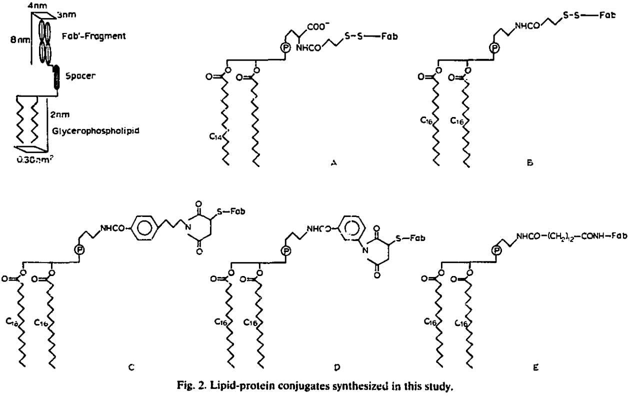

Fig. 2. Lipid-protein conjugates synthesized in this study.

lipid, whereas the dithiopropionyl conjugate is cleavable with reducing agents, allowing control experiments. Texas red-labelled $F(ab')_2$ fragments were selectively cleaved into monovalent Fab-fragments of about 50 kDa by reduction with dithiotreitol (DTT). $F(ab')_2$ fragments (10 mg/ml) were transferred to deoxygenated 100 mM citrate, 100 mM NaCl, 2 mM EDTA buffer. Fresh DTT solution was added to a final concentration of 20 mM. The pH was lowered to pH 4.8 in order to keep the intrachain and heavy/light chain disulfide-bonds intact. After incubating for 90 min at room temperature under an argon atmosphere with occasional shaking, the DTT was removed by chromatography on a Sephadex G-25 column, equilibrated in deoxygenated Hepes buffer. To avoid, that upon removal of DTT the Fab-monomers re-anneal to $F(ab')_2$ fragments, the freshly reduced Fab fragments were immediately brought into contact with liposomes with and without spacer-lipids. The pH was adjusted to pH 8.0 for pyridyldisulfides and pH 6.8 in case of the maleimides followed by flushing the vials with argon. The coupling reaction was performed in deoxygenated buffer for 14 h at room temperature in the former and for 6 h at 30°C in the latter case. A molar ratio of 0.05 of spacer-lipid to the matrix lipid DMPC and protein concentrations of approx. 2 mg Fab-fragments per μmol of total lipid were used throughout the crosslinking reactions and yielded satisfactory coupling efficiencies (Table I).

When coupling antibody fragments to carboacyl derivatives of DPPE, the antibodies were cleaved by papain [22] and EDC coupling reagent was used. The reaction was performed according to the procedure of Kung [21] except that 1.0 mg Fab fragments per μmol total lipid were reacted at 4°C and pH 8.0 for 12 h. The conjugated liposomes were finally separated from unbound Fab-fragments through density gradient centrifugation on metrizamide (Beckman Ti 45 Rotor for 8 h at $300000 \times g$). Protein was measured by a modified Lowry method [23]. The phospholipid concentrations of the resulting liposome suspension were determined directly by a lipid phosphorous measurement after extraction of the lipid into chloroform [24]. In the case of dye-labelled proteins, concentrations were calculated from absorption measurements at 596/280 nm. Purified F_{ab}-lipid vesicles which did not find an immediate use in the experiments were slowly cooled down to 4°C and then stored under liquid nitrogen.

Preparation of planar supported membranes from liposomes containing Fab-lipids. The procedure of spreading a monolayer from a suspension of Fab-lipid vesicles (see Fig. 1) was carried out as described by Heyn et al. [25]. The experimental setup is represented in detail by Heyn et al. [26]. Briefly a monolayer was allowed to form in a spreading well by spontaneous selfassembly from the vesicle suspension at the air/water interface. To separate the monolayer from attached vesicles and other bilayer-structures the spreading well communicated with a Langmuir-Blodgett (LB) trough through a thin buffer film which was supported by a wet stripe of cleaned filter paper (Schleicher & Schuell). Due to the gradient in lateral pressure the monolayer expanded over the wet bridge and was thus transferred onto the subphase of the adjacent trough. After the removal of the wet-bridge, the thermodynamic state of the monolayer could be controlled and observation was possible by the fluorescence microscope. Eventually the monolayer could be transferred by the horizontal dipping technique [27] onto a solid support pre-coated with covalently linked hydrocarbon chains. Alkylation of quartz or mica with octadecyltrichlorosilane (OTS) was carried out as described by Moaz et al. [28]. The contact angle of a progressing drop of water was measured to be greater than 110°.

Site specific coupling of Fab-fragments to a preformed lipid monolayer. Direct crosslinking of Fab-fragments via disulfide bonds to preformed monolayers was done in a fast three step procedure (see also Fig. 1). In the first step a lipid monolayer containing the spacer-lipid PDP-PE and the filling lipid DMPE in a molar ratio of 0.4 were spread from a chloroformic solution at the air/water interface. A small amount (typically 1%) of fluorescent-labelled lipid (NBD-PC) allowed the observation of the phase separation and domain formation while compressing and reducing the surface area in the Langmuir trough. After the typical crystal pattern had formed, the monolayer was transferred at 28 mN/m onto an alkylated quartz slide (see above). This preformed monolayer was kept submerged in a special compartment which was then removed from the Langmuir trough for the crosslinking reaction with Fab-fragments. In the second step the aqueous solution (approx. 50 μl) which was retained in the small compartment, was slowly exchanged against a solution of freshly reduced F_{ab}-fragments (1.5 mg/ml) in deoxygenated Hepes buffer (no salt added). The system was kept in a humid argon atmosphere at 37°C for 20 min. During this time the solution containing Fab-fragments was renewed three times. In the last step reactive sites were blocked with fresh 0.15 M iodoacetamide solution and unreacted material was carefully washed out with an excess of high salt Hepes buffer (0.5 M NaCl).

Differential scanning calorimetry. Samples were prepared for differential scanning calorimetry as described by Heise et al. [29]. DSC spectra were obtained on a MC-2 micro-calorimeter (Microcal, Amherst, USA) in the hs-mode operated from an IBM compatible computer with DA-2 Microcal software.

Enzyme linked immunosorbent assay. The ELISA procedure is taken from Leahy et al. [30]. It was applied to whole antibodies from cell culture supernatents, antibody fragments and conjugates with the following modification. Polystyrene plates (Costar A/2) were passively coated with DNP-BSA or DNP-OA (50 μg/ml) in 20 mM Tris, 500 mM NaCl (pH 7.5) (TBS buffer). Carrier protein without hapten and pure DMPC liposomes were used as negative control. The wells were blocked with 3% gelatin, washed three times with the same buffer and flicked dry. Appropriately diluted supernatant or Fab-lipid suspension (in TBS/1% gelatin) was added to the wells and incubated for 1 h at 37°C. The plates were washed with TBS buffer/0.05% Tween 20 and specific binding activity was detected with affinity purified horseradish peroxidase conjugated rabbit anti-mouse IgG (Dakopatts, Hamburg, FRG). 4-Chloro-1-naphthol (Bio-Rad, Richmond, USA) in 50 mM TBS, containing 0.015% H_2O_2as oxidizing agent, was used as chromogene.

Dual fluorescence binding assay. Emission spectra of liposomes with covalently coupled monoclonal Fab-fragments exclusively carrying the fluorescent dye TxR were measured in a spectrofluorometer (Fluorolog-2, Spex, NJ, USA) at an excitation wavelength of 595 nm while slowly stirring. A suspension of disulfide-linked Fab-vesicles was subsequently brought in contact with OA, which was labelled with an average of 10.5 FITC molecules and two DNP haptens. This low hapten concentration was chosen to avoid extensive agglutination of liposomes and precipitation. The final concentration of FITC-DNP-OA was 0.25 mg/ml. After 30 min at 25°C with stirring not specifically bound material was separated by density gradient centrifugation as described above. The band with the liposomes was subtracted from the centrifugation tube and diluted to the same concentration as before. Dual fluorescence emission was detected for the corresponding excitation wavelengths. Finally the Fab-lipid vesicles were incubated 20 min at 25°C at pH 7.8 with 50 mM DTT. Following repeated centrifugation and separation, the fluorescence emission was determined again. This time the fluorescence of the protein band, which separated at a higher density, was measured additionally.

Microfluorimetry using supported planar membranes. We performed binding experiments with the planar, Fab-lipid containing monolayer on alkylated glass. The sample with the immobilized monolayer was kept in a small compartment of typically 50 μl volume for transfer to further experiments. A fluorescence microscope on an *xy*-stage allows the determination of the lateral distribution of fluorescent markers in the monolayer. Recordings were made using a SIT camera interfaced to an image analyzing system. The local fluorescence intensity was measured from a 20 μm spot using an attached microfluorimeter [26]. The sample was incubated with BSA carrying an average of 5 DNP haptens and 8 fluorescein groups per BSA molecule either in a 10-fold excess of unaltered BSA or after previous blocking with 1% (w/v) BSA. The antigen concentration varied from 75 mM down to 30 nM. The chamber was kept at 37°C for 20 min in a humid atmosphere. Unbound material was removed by washing the chamber with 1 ml 10 mM Hepes buffer with 0.5 M NaCl. As a negative control, fluorescein-labelled BSA lacking DNP groups was applied in the same procedure. In order 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 was used on both, antigen and Fab-lipid. In this case the measured fluorescence ratio with and without antigen was (with the known fluorophore content of the carriers) directly convertible to a ratio of amount of bound antigen to number of binding sites. The fluorescence was averaged over 20 spots in three samples and normalized by the number of labels per molecule with the Texas red signal of the protein-rich region set to 100%.

Results and Discussion

Coupling of monoclonal anti-DNP Fab-fragments to phospholipids was achieved trough three heterobifunctional reagents and EDC, providing different covalent bonds and crosslinking spacer lengths. The goal was to maintain a maximum of accessibility and activity of the binding sites. A site specific linkage at the opposite side of the antigen binding pocket appeared to be most suitable. As has been shown by various investigators, this type of coupling between lipid and protein can be performed in vesicle suspensions. The major advantage compared to reactions in detergent suspensions is, that no reconstitution into bilayer systems is required any more avoiding potential problems with imperfect re-

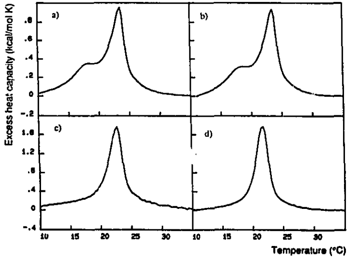

Fig. 3. Differential scanning calorimetry curves of DMPC vesicles containing 5% of the reactive spacer-lipids: (a) DTP-PE (lipid B) and (b) MB-PE (lipid D). (c) and (d) show the corresponding scans after reaction with the Fab-fragments.

moval of detergent or randomly orientated insertion of the proteins into the membranes. Following the given procedures we have synthesized five different molecules (Table I and Fig. 2). The length of the lipid moiety was chosen so that the fluid/solid phase transition occurs in a temperature range where the protein moiety remains intact. The reactive groups were chosen to compare nonspecific coupling to free amines on the protein with the site-specific reaction to the sulfhydryl group. The coupling efficiencies are listed in Table I together with the controls for nonspecific adsorption of the Fab-fragments to the vesicles. Vesicles containing lipid E clearly showed the highest amount of Fab-fragments bound to the vesicles but a significant fraction of the Fab-fragments turned out to be non-specifically adsorbed. Therefore, molecule E was not used for further experiments. Lipids A and B gave about equally high coupling efficiencies and showed only very minor nonspecific adsorption.

In order to further characterize the resulting Fab-lipids and their interaction with the lipid moiety of the lipid matrix, differential Scanning Calorimetry (DSC) experiments were carried out. Figs. 3a and 3b show the phase transitions of DMPC vesicles containing 5% of the lipids B and D. The main transition occurs at 23.5°C and 23. 6°C, respectively. The phase transition appeared slightly broadened and shifted compared to pure DMPC (23.8°C). The transition enthalpies $H_0 = 5.5 \pm 0.5$ and 5.0 ± 0.5 kcal/mol for samples containing lipids B and D, respectively, are slightly reduced compared to pure DMPC ($H_0 = 6.5$ kcal/mol). This is what one could expect as an effect of the minor fraction of the spacer-lipid. The second characteristic feature in both DSC scans is a broad shoulder at lower

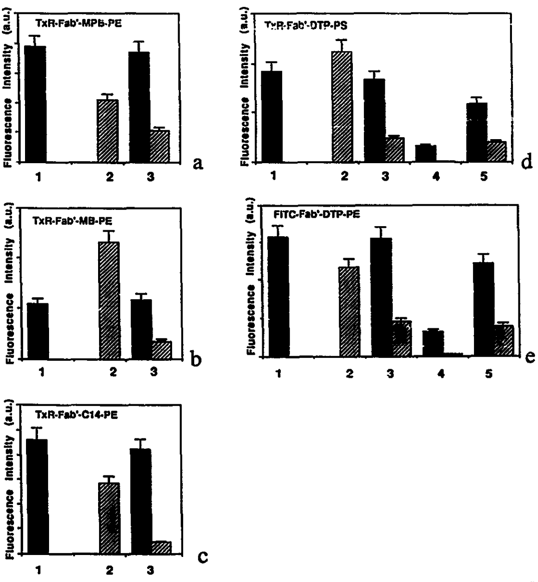

Fig. 4. Column 1: Emission intensities of the different fluorescence labelled Fab-lipids A–E in figures a–e, respectively. Column 2: Emission intensities of the labelled antigen solution. Column 3: Intensities of labelled Fab-lipids A–E and antigens after incubation and washing. Column 4: Intensities measured from the vesicles (like Column 3) but after cleaving the disulfide bond between Fab-fragments and lipid and after separation from the supernatant. Column 5: Intensities from the supernatant.

temperatures which was stable for several heating and cooling cycles. Such shoulders are known to occur with small sonicated vesicles, but this effect usually relaxes after the first scan and shows a different line shape [31]. Therefore, this feature might reflect the interaction of the spacer arms with the matrix lipid.

After the coupling reaction with the Fab-fragments (Figs. 3c,d) the above shoulder was no more detectable and the main transition temperature was significantly shifted to 22.8 and 21.8 °C (Fab-lipids B and D, respectively). However, the transition remained sharp. The corresponding enthalpies were slightly increased to 6.0 ± 0.5 and 5.8 ± 0.5 kcal /mol for molecule B and D, respectively. It is well known that the phase transitions of lipid bilayers can be influenced drastically by interaction with proteins. In our case where we have a molar ratio of Fab-lipid to matrix lipid in the order of 1:500, each Fab-fragment can be in direct contact with nearly all lipid molecules taking a circle of 200 Å diameter for the Fab-fragment and an average area of 70 $Å^2$ per lipid. As the phase transition is not broadened but just shifted to lower temperatures we have no evidence for a phase segregation so that we would conclude that the Fab-lipids are evenly distributed at the surface of the vesicles and that there is no strong interaction between the matrix lipid and the protein moiety. The latter effect might be due to the comparably long spacer arms which may keep the proteins at a distance of about 10 Å away from the vesicle surface.

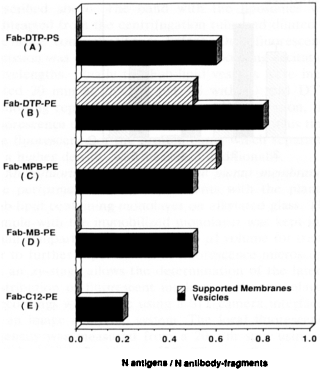

Fig. 5. Binding activity of the different Fab-lipids on vesicles and supported planar membranes. Binding to molecules A. D and E was measured on vesicles only.

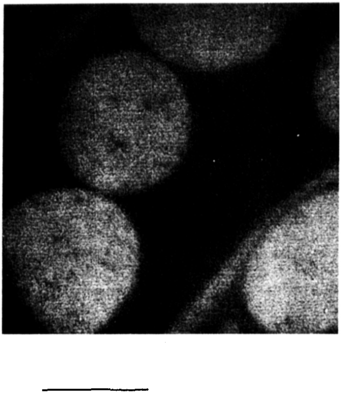

Fig. 6. Fluorescence micrograph showing the two-dimensional domain pattern of a monolayer at the air/water interface formed from Fab-lipid vesicles at a lateral pressure of 30 mN/m at room temperature.

In order to ensure that the binding site of the protein remained active in our hybrid molecules, dual fluorescence binding assays were performed for all five molecules. The experiments are listed in Fig. 4 and the corresponding binding activities are summarized in Fig. 5. Here Fab-lipid B scored best when binding was measured on the vesicles. The high binding efficiency of 80% (see Fig. 5) strongly suggests, that the Fab-fragment is bound in a highly oriented manner with the binding pocket pointing away from the membrane surface. With the given size of the antigen, binding would be sterically hindered for all other orientations of the Fab-fragment. It also shows that only a minor fraction of the binding sites is either not accessible or inactive. In the case of the second best binding performance (Fab-lipid C) the active fraction is still 50% making it a feasible candidate for further experiments, too. An additional check for the specificity of the binding was carried out with molecules A and B. Here the hapten-Fab complexes were cleaved from the lipid by reduction of the disulfide bond and removed from the vesicles. Fluorescence intensities from the labels on the haptens and on the Fab-fragments were measured both from the remaining vesicles and from the removed complexes. The corresponding results are listed in Figs. 4d and 4e in columns 4 and 5 and show that under

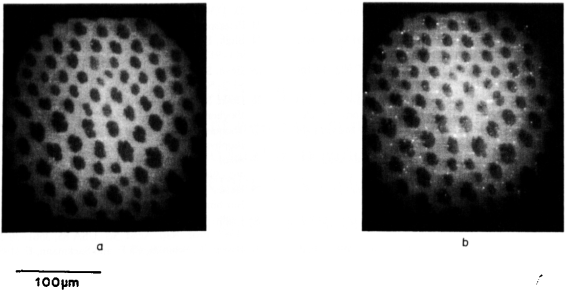

Fig. 7. (a) Fluorescence micrograph of a supported planar membrane consisting of the matrixlipid DMPE containing 30 mol% spacer-lipid A and a fraction of 1% NBD-labelled PC which is excluded from the dark crystals. This image was recorded in green emission. (b) Same area after reacting Texas red-labelled Fab-fragments seen in red emission.

these conditions hapten and Fab-fragment are removed as a complex.

Vesicles containing Fab-lipids B and C were then used to form monomolecular films at the air/water interface. As we have shown in detail elsewhere [32] such films can be separated from attached vesicles and driven into a two-dimensional phase segregation. The resulting pattern can be visualized by fluorescence microscopy at the air/water interface and is shown in Fig. 6. Both phases, the bright protein-rich and the dark lipid phase, seem to be fluid-like phases as judged from the observed shape fluctuations. We saw no significant difference in the domain shapes between the two different Fab-lipids which were studied. Only a slight difference in binding activity was observed for films transferred onto solid supports. The results are also listed in Fig 5.

The above given protocol describes a very useful method for the formation of supported planar membranes from vesicles containing lipid-anchored proteins. The possibility to image such membrane-bound proteins by Atomic Force Microscopy has been reported recently [33]. However, for the formation of recognition pattern on solid supports like the surface of any kind of sensors, a simpler method would be desirable. We therefore have investigated also the potential to couple the Fab-fragments to a preformed pattern of reactive spacer-lipids embedded in an inert lipid matrix. In Fig. 7 such a pattern is shown in a fluorescence micrograph. It was created by first allowing a mixed DMPE/spacer-lipid A monolayer to form DMPE crystals, here shown as dark domains. The Fab-fragments were then covalently bound to the reactive spacer-lipids after the film had been transferred onto a silanized quartz slide in a way that the pattern stayed preserved. As the spacer-lipid was excluded from the growing crystals, the remaining surface is now covered with bound Fab-fragments, forming a selective recognition pattern for the corresponding antigen. The major advantages of this approach are not only in technical feasibility but also in the broad variety of pattern that can be obtained ranging from a stripe pattern [34] with spacings down to far below 1 µm up to hexagonally packed superlattices [35] with dimensions up to some 100 µm. The use of polymerized membranes for increased mechanical stability may broaden the scope of potential applications [36].

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