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Use of an Oriented Transmembrane Protein to Probe the Assembly of a Supported Phospholipid Bilayer

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ABSTRACT Planar-supported phospholipid bilayers formed by the adsorption of vesicles are increasingly used in the investigation of lipid-dependent reactions. We have studied the way in which these bilayers are formed with phospholipid vesicles containing the transmembrane protein Tissue Factor (TF). TF complexed with the serine protease, factor VIIa, is the primary initiator of blood coagulation by way of activation of the zymogen factor X. TF has been shown to orient randomly on the inner and outer leaflets of vesicles. We used proteolytic digestion to produce vesicles in which the extracellular domain of TF is located on the inner leaflet. These vesicles show no cofactor activity for factor VIIa as a result of the inability of the extracellular domain of TF to bind VIIa. After freeze/thawing, 50% of the cofactor activity was regained, indicating reorientation of the sequestered, inner leaflet TF. Adsorption of these vesicles to the inner surface of glass microcapillaries results in a continuous phospholipid bilayer. The microcapillaries were perfused with a solution of factors VIIa and X, and the effluent was monitored for factor Xa production, a sensitive measure of the activity of the TF-VIIa complex. For coatings produced with the digested vesicles, minimal TF-VIIa activity was observed, showing that the supported bilayer preserves the orientation of the leaflets in the vesicles, i.e., the outer leaflet of the vesicles forms the outer leaflet of the supported bilayer.

INTRODUCTION

Supported phospholipid bilayers have gained considerable importance as model systems for the study of biological membranes and lipid-dependent reactions. These bilayers have been produced on a wide variety of surfaces such as glass, quartz, silicon, mica, platinum, and chromium (Tamm and McConnell, 1985; Helm et al., 1989; Tien and Salamon, 1990; Cuypers et al., 1983). Planar bilayers on solid surfaces are frequently produced by the use of Langmuir-Blodgett techniques (Agarwal, 1988), but have also been produced by adsorption of phospholipid vesicles to glass substrates (Watts et al., 1984). These two methods have been shown to produce bilayers that are physically and biologically indistinguishable from each other (Giesen et al., 1991). The latter method has the advantages of allowing the incorporation of proteins into the membranes and the use of surfaces of varied geometry, such as spherical beads (Bayerl and Bloom, 1990) and cylindrical tubes (Gemmel et al., 1988). The physical properties of these bilayers and their interaction with the support surface have been well studied, but the process by which phospholipid vesicles assemble to form a continuous supported bilayer is not well understood.

In forming a continuous supported bilayer, it is unknown whether a vesicle retains a unique or a randomly deposited orientation. We have used the orientation of a transmembrane protein to probe the assembly of a supported phospholipid bilayer. The protein used in these studies is Tissue Factor (TF), a glycoprotein consisting of an extracellular domain (residues 1–219), a single transmembrane domain (residues 220–242), and a cytoplasmic domain (residues 243–263). After tissue damage, the binding of factor VIIa, a serine protease, to TF produces a proteolytic complex that is the primary initiator of blood coagulation (Nemerson, 1988). This complex activates factor X to Xa. The production of Xa was used as a sensitive measure of TF activity. Upon incorporation into phospholipid vesicles, TF was shown to orient randomly on the inner and outer leaflets (Bach et al., 1986). Proteolytic digestion of TF on the outer leaflet produced vesicles that were inert as a cofactor for the enzyme VIIa. Upon randomization of the vesicles by repeated freezing and thawing, the activity of the TF located on the inside can be regained (see Fig. 1). We used these vesicles to characterize the assembly of supported bilayers on the inner surface of glass microcapillary tubes. The data in this paper indicate that assembly of the bilayer occurs with a specific orientation such that the outer leaflet of the vesicles forms the outer leaflet of the supported bilayer.

MATERIALS AND METHODS

1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine (DOPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. β-octyl-glucopyranoside (βOG) was purchased from Calbiochem (La Jolla, CA).

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Abbreviations used: DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; βOG, β-octyl-glucoside (octyl-pyranoside); IPR-pNA, Ile-Pro-Arg-*p*-nitroaniline; chromogenic substrate for Xa; DFP, diisopropyl fluorophosphate; HBS, HEPES-buffered saline (10 mM HEPES; 0.14 M NaCl; pH 7.5); HBS-BSA, HBS with 1.0 mg/ml bovine serum albumin; EDTA-BSA, 50 mM EDTA; 0.5 mg/ml BSA; pH 7.5; ^{3}H-TF, tritium-labeled recombinant human tissue factor; ^{14}C-DOPS, radiocarbon-labeled DOPC; LSC, liquid scintillation counting.

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Human coagulation factor VIIa was purchased from Novo Nordisk A/S 2880 (Hilleroed, Denmark), and human factor X was purified as previously described (Miletich et al., 1991). Recombinant human Tissue Factor (TF) in βOG was a generous gift from Dr. Gordon Vehar, Genentech, Inc. (South San Francisco, CA). Diisopropyl-fluorophosphate (DFP) and Subtilisin (Protease Type XXIV) were purchased from Sigma Chemical Co. (St. Louis, MO). ^{14}C-DOPC was purchased from Amersham (Arlington Heights, IL). Aquasol-2 scintillation cocktail was purchased from Dupont-NEN Research Products (Boston, MA). The chromogenic substrate for factor Xa; Ile-Pro-Arg-*p*-nitroaniline (IPR-pNA) was synthesized by our laboratory. ^{3}H-TF was prepared by the reaction of TF with succinimidyl [2,3-^{3}H] propionate as per the manufacturers instructions (Dupont-NEN Research Products). Specific activity of ^{3}H-TF was 4.3×10^{6} cpm/μg.

Reconstitution of TF into phospholipid vesicles

DOPS and DOPC in $CHCl_3$ were combined to yield a molar ratio of 30% DOPS and 70% DOPC (using manufacturers weights) and dried under N_2 for 2 h onto the walls of a borosilicate glass tube. The lipids were then placed in vacuo overnight to ensure complete drying. A solution of 375 mM βOG in HEPES-buffered saline (HBS) (0.01 M HEPES; 0.14 M NaCl; 0.01% NaN_3, pH 7.5) (≈1 ml) was added to the dried lipids to yield a final lipid concentration of 25 mM. TF was added to give the desired molar ratio of protein-to-lipid (typically 1/100,000). Tracer amounts of ^{14}C-DOPC and ^{3}H-TF were added for precise quantification of protein and lipid concentrations in the final material. The preparation was gently vortexed at room temperature until the lipids were solubilized. After completely dissolving the lipids, an aliquot was taken for liquid scintillation counting (LSC) and the remaining solution transferred to dialysis tubing (12,000–14,000 molecular weight cutoff; SpectroPor). The preparation was dialyzed against 3 × 1 l of HBS for 72 h, with buffer changes every 24 h. After dialysis, an aliquot was again taken for LSC and the remaining solution was applied to a 1.0 × 60 cm Sepharose CL-2B (Pharmacia, Piscataway, NJ) column equilibrated with HBS. Column fractions were collected, and the elution profile was obtained by light scattering at 280 nm. The elution peak was pooled, and the lipid and protein concentrations were determined radiometrically by comparison with the starting material. Vesicle preparations were stored under N_2 and refrigerated when not in use. Vesicles without TF were prepared as described above with the omission of protein and protein tracer.

Proteolytic digestion of tissue factor in vesicles

A sample of TF:DOPS:DOPC vesicles was mixed with DOPS:DOPC vesicles to a TF concentration of 10 nM (lipid was maintained at 2 mM). This sample was freeze/thawed before use to assure fully randomized vesicles. Freeze/thawing consisted of five cycles of freezing in a mixture of acetone/dry ice and thawing in a water bath at 37°C. Concentrated protease solutions were made just before use and kept on ice (15 mg of subtilisin to 0.5 ml HBS). Digestions were started by addition of protease (30 μl) to a solution of TF vesicles (270 μl of TF:DOPS:DOPC (30:70) and incubated at 37°C for 3 h. The protease was then inactivated by the addition of 1 μl of DFP (2.5 M DFP in 2-propanol) for 1 h at 37°C. A control sample was treated identically except for the addition of an equivalent amount of buffer in place of the protease. The digested sample was then divided equally, and one portion was freeze/thawed.

Tissue factor activity assays

Static assays

An automated robotic instrument has been developed by our laboratory to perform kinetic assays of TF activity. TF samples were added to Eppendorf tubes containing factors VIIa (10 nM) and X (250 nM) in HEPES-buffered saline with 1 mg/ml bovine serum albumin (HBS-BSA) at 37°C. Final TF concentrations were in the range of 1–10 pM. The reaction was initiated with the addition of Ca^{2+} (5 mM). Aliquots were taken at timed intervals and diluted into the wells of ELISA plates containing 125 μl of 50 mM EDTA and 0.5 mg/ml BSA, pH 7.5, which quenched the reaction. Factor Xa concentrations were quantified by the addition of chromogenic substrate, IPR-pNA, to a final concentration of 1.5 mM. The absorbance at 405 nm was measured for 10 min at 37°C using a kinetic ELISA plate reader (TMax, Molecular Devices, Menlo Park, CA). The formation of factor Xa in time was fit by linear regression and used as the measure of TF activity.

Continuous flow reactor assays

The instrumental setup for the automated flow reactor system was as described previously, (Contino et al., 1991). Cleaned microcapillary tubes (0.27 mm inner diameter × 128 mm) were filled with the appropriate TF-phospholipid vesicles at room temperature and incubated for 20 min. For tubes coated with control vesicles, the TF surface density was ≈1.3 fmol/cm^2. Thereafter, the tubes were flushed with HBS-BSA for 8 min at a wall shear rate of 3000 s^{-1}. The coated tubes were then perfused with a reaction mixture containing factors VIIa (10 nM), X (600 nM), and Ca^{2+} (5 mM) in HBS-BSA at a wall shear rate of 1600 s^{-1} at 37°C. The perfusate from individual tubes was collected at timed intervals into the wells of ELISA plates containing 75 μl of 50 mM EDTA and 0.5 mg/ml BSA, pH 7.5. The plates were assayed as described above for factor Xa production. The Xa production curve was integrated over the full time course of 24 min and used as the measure of TF activity.

RESULTS AND DISCUSSION

Proteolytic digestion and freeze/thawing of TF vesicles was used to produce three types of vesicles: control, which have TF equally distributed on the inner and outer leaflets; digest, TF only on the inner leaflet; and Digest Freeze/Thaw, which are TF-digested vesicles that have been randomized by the process of freeze/thawing (see Fig. 1). Static assays for TF activity show that proteolytic digestion of the outer leaflet TF is complete. Upon freeze/thawing, TF is randomized on the inner and outer leaflets of the vesicle (see Table 1). The regained activity after freeze/thawing demonstrates that the digestion has no effect on TF on the inner leaflet. To deduce the orientation of vesicle deposition in the formation of a supported bilayer, we used these three kinds of vesicles in the continuous flow reactor and monitored TF activity.

We can envision three ways in which a vesicle is adsorbed at the support surface. One, the outer leaflet is adsorbed to the surface preferentially exposing the inner leaflet. Two, the adsorbed vesicle breaks open at its contact with the support surface, and the inner leaflet is adsorbed to the surface preferentially exposing the outer leaflet. Three, the vesicle is adsorbed in a random fashion. After digestion of the extracellular domain of TF on the outer leaflet, oriented deposition of these vesicles should result in supported bilayers with either 100% or no activity compared with the control (see Fig. 2). Random deposition would result in an intermediate activity.

Table 1 demonstrates that TF activity in bilayers formed from digested vesicles is less than 7% of the control activity, which suggests a predominant orientation to the vesicle deposition on the glass surface. As expected, the Digest Freeze/Thaw presented 50% of the control activity, which mirrors the results found for vesicles in the static assays. Fig. 3 shows the typical profiles generated in the continuous flow reactor.

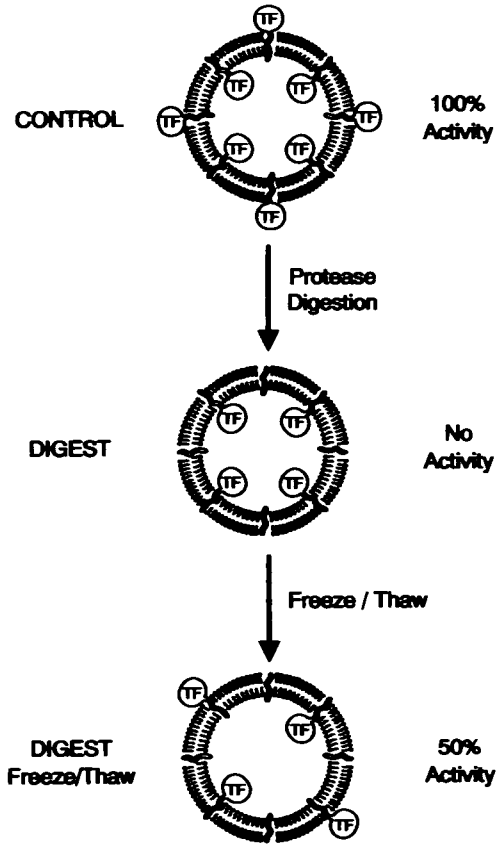

FIGURE 1 Schematic representation of Tissue Factor: phospholipid vesicles. The CONTROL vesicles are shown with TF randomly distributed on the inner and outer leaflets. Upon treatment with protease, the outer leaflet TF molecules are digested, leaving the remaining TF molecules sequestered within the vesicle (DIGEST). By repeated freeze/thawing of these vesicles, the orientation of the remaining TF molecules can be randomized (DIGEST Freeze/Thaw). The TF:VIIa activity of the DIGEST and DIGEST Freeze/Thaw vesicles are expected to be 0 and 50%, respectively, as compared with the CONTROL vesicles.

TABLE 1 Comparison of tissue factor activity in static and continuous flow assays

	Factor Xa production			
	Static*		Flow‡	
	pmol/min	% of control	pmol	% of control
Control	59.0 ± 1.3	100	130.9 ± 1.1	100
Digest	0.2 ± 0.1	0.4 ± 0.1	9.1 ± 1.0	6.9 ± 0.7
Digest freeze/thaw	28.5 ± 0.8	48.2 ± 0.5	60.4 ± 3.7	46.1 ± 2.7

Data are presented as the mean ± C.V. of four experiments.

* Static: Samples of the control, digest, and digest freeze/thaw vesicles diluted to 10 pM TF were incubated with 10 nM factor VIIa and 250 nM factor X in HBS-BSA. The reaction was initiated with 5 mM Ca^{2+}. Factor Xa production was measured in timed aliquots.

‡ Flow: Glass microcapillaries were filled with the indicated vesicle solution and then flushed with HBS-BSA. The coated tubes were perfused with a solution containing 10 nM factor VIIa, 600 nM factor X, and 5 mM Ca^{2+} in HBS-BSA at a wall shear rate of 1600 s^{-1} at 37°C. The perfusate was collected into the wells of an ELISA plate filled with an EDTA solution. The data presented are the integrations of the Xa production curves over the full time course (see Fig. 3).

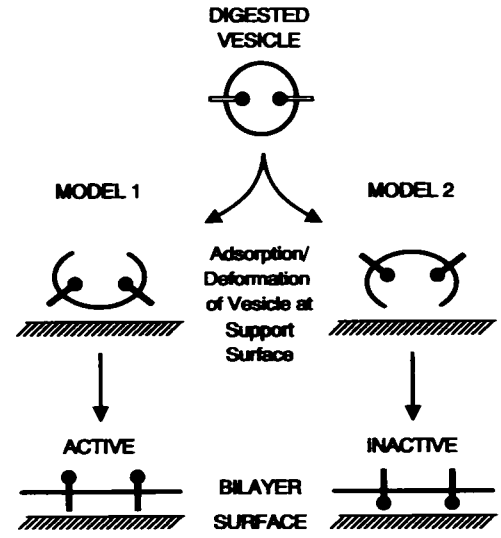

FIGURE 2 Schematic representation of vesicle deformation and adsorption to the support surface. Model 1 shows the vesicle deposition with the outer leaflet oriented towards the surface. Model 2 shows the adsorption of the inner leaflet. The TF of the digested vesicles by model 1 will be 100% active, whereas by model 2 all activity will be absent.

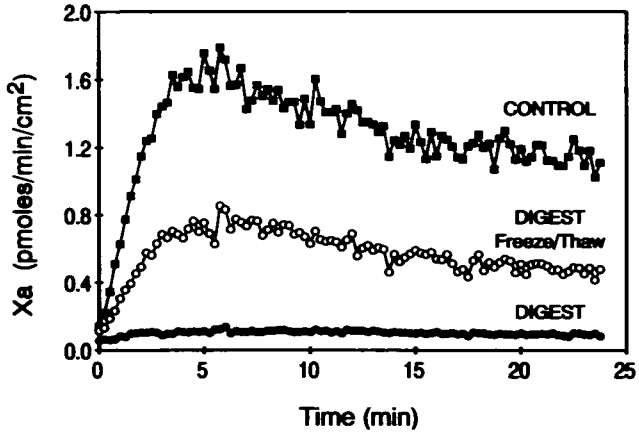

FIGURE 3 Xa production curves for the three types of vesicles in the continuous flow reactor. The glass microcapillary tubes were filled with the indicated vesicle preparation for 20 min at room temperature. The tubes were flushed with HBS-BSA for 8 min at a wall shear rate of 3000 s^{-1}. The reaction was initiated by perfusion of the tube with a solution containing 600 nM factor X, 10 nM factor VIIa, 5 mM Ca^{2+} at a wall shear rate of 1600 s^{-1} at 37°C. Each curve is typical of four separate experiments.

The process by which a phospholipid bilayer is deposited on a solid surface is not well understood. It involves transport of the vesicles towards and adherence to the surface, deformation and rupture of the vesicles, straightening of the phospholipid surfaces, and fusion of these surfaces. The adherence of negatively charged DOPS-containing vesicles to a negatively charged glass surface requires the presence of counter ions. Interestingly, the deposition of phospholipid stops at a single bilayer (Cuypers et al., 1983; Giesen et al., 1991; Gemmel et al., 1988; Contino et al., 1991; Schoen et al., 1990), which is separated from the support surface by a 1.7 ± 0.5 nm water layer (Bayerl and Bloom, 1990; Andree

et al., 1992). Apparently, phospholipid vesicles do not adhere to preformed bilayers. The question we address here is whether there is a sidedness to the deposition process. Kalb and co-workers (Kalb et al., 1992; Kalb and Tamm, 1992) have used vesicle fusion to supported monolayers to produce supported bilayers. They have proposed a mechanism that results in both the inner and outer leaflets of the vesicle forming the second monolayer. This situation is significantly different from the one we use, in that vesicle fusion to a preformed monolayer is driven by hydrophobic interactions, whereas fusion to a glass surface is largely hydrophilic. We have shown that the supported bilayers formed on glass surfaces by phospholipid vesicles with the extracellular domain of TF located only on the inner leaflet have little cofactor activity for VIIa. We have also shown that control vesicles with the extracellular domain located on both surfaces maintain cofactor activity for VIIa. Together, these results indicate that the vesicle deposition has a predominant orientation in which greater than 93% of the vesicles are directed toward the inner leaflet of the support surface. Moreover, the design of the experiment demonstrates that the protein plays no role in establishing the orientation of the supported phospholipid bilayer.

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