Coalescence of phospholipid membranes as a possible origin of anticoagulant effect of serum proteins

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Abstract

Interactions between phospholipid membranes (made of palmitoyloleoylphosphatidylcholine, cardiolipin and cholesterol) after addition of β2 glycoprotein I (β2GPI) or anti-β2GPI antibodies or a mixture of both were studied by observing giant phospholipid vesicles under the phase contrast microscope. Both, negatively charged and neutral vesicles coalesced into complexes and adhered to the bottom of the observation chamber in the presence of β2GPI in solution. Anti-β2GPIs alone or previously mixed with β2GPI caused coalescence of charged but not neutral vesicles, i.e. for neutral membranes the effect of β2GPI was abolished by the presence of anti-β2GPIs. Since the presence of the above adhesion mediators can prevent fragmentation of the membrane we propose a (new) possible anticoagulant mechanism for some serum proteins by preventing the release of prothrombogenic microexovesicles into circulation.
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1. Introduction

In spite of considerable knowledge gathered on the role of complex interactions between phospholipids, β2GPI and antiphospholipid antibodies in thrombosis and haemostasis the underlying mechanisms are not yet completely understood. However, these interactions can be studied also in relatively simple model systems, such as aqueous solution of giant phopsholipid vesicles (GPVs) with exogeneously added β2GPI and/or antiphospholipid antibodies.

The serum protein β2GPI is considered to exhibit a variety of physiological roles, among them in the process of blood clot formation. It was found

that it affects the metabolism of triacylglycerol-rich lipoproteins, the function of thrombocytes, and the activation of endothelial cells (Bevers et al., 2005 and references therein). Moreover, β2GPI inhibits the transformation of prothrombin into thrombin (Nimpf et al., 1986). It binds to structures which contain negatively charged phospholipid molecules, such as thrombocytes (Schousboe, 1980), thrombocyte-derived microvesicles, apoptotic cells (Price et al., 1996), and serum lipoproteins (Polz and Kostner, 1979; Kobayashi et al., 2003). Further, it mediates cellular recognition of negatively charged phospholipid-exposing microparticles (Balasubramanian et al., 1997; Moestrup et al., 1998; Thiagarajan et al., 1999), thereby indicating its anticoagulant role in the clearance of procoagulant negatively charged microvesicles from the circulation (Zwaal, 1978; Aupeix et al., 1996; Zwaal et al., 1997; Sabatier et al., 2002; Greenwalt, 2006; Chonn et al., 1995; Hanczyc and Szostak, 2004).

Antiphospholipid antibodies are found in the blood sera of patients with antiphospholipid syndrome—a disorder which is connected with arterial and venous thrombosis, fetal loss and thrombocytopenia (Roubey, 1996; Levine et al., 2002). Antiphospholipid antibodies were indicated to inhibit protein C activation (Hasselaar et al., 1989), while the binding of antiphospholipid antibody–β2GPI complexes to cell surfaces was found to promote activation of thrombocytes (Khamashta et al., 1988) as well as endothelial cells in vitro (Simantov et al., 1995; Del Papa et al., 1995) and in vivo (Pierangeli et al., 1999).

In a previous study (Ambrožič et al., 2005), individual GPVs were transferred to a solution containing β2GPI and/or monoclonal anti-β2GPI antibodies. The budding of phosphatidylserine-containing GPVs was induced by addition of both, β2GPI and by mixtures of β2GPI with monoclonal anti-β2GPI antibodies, while monoclonal anti-β2GPI antibodies alone had no effect. In observing collective effects, the coalescence of negatively charged cardiolipin-containing vesicles was induced by a patient's IgG (the most abundant immunoglobulin in plasma) fractions containing antiphospholipid antibodies and/or β2GPI (Ambrožič et al., 2006). In the present work, these interactions were further studied on dispersions of negatively charged and neutral GPVs, budding GPVs with β2GPI, monoclonal anti-β2GPI antibodies and the IgG fraction of a patient with antiphospholipid syndrome as mediators.

2. Methods

2.1. β2GPI and antiphospholipid antibodies

β2GPI (Hyphen BioMed, Neuville-sur-Oise, France) was aliquoted and stored at −70 °C. In all experiments, the final concentration of β2GPI in phosphate buffer saline (PBS) was 100 mg/l, which is approximately half the concentration of physiological β2GPI in normal human plasma (about 200 mg/l) (Polz and Kostner, 1979; McNally et al., 1993).

Monoclonal HCAL anti-β2GPI antibodies (HCALaβ2GPI) (chimeric IgG monoclonal anti-β2GPI antibodies, consisting of constant human and variable mouse regions), obtained from BALB/c mice (an albino strain of laboratory mouse) immunized with human β2GPI (Igarashi et al., 1996) and recognizing domain V of β2GPI (C-terminal domain of β2GPI carrying a positively charged lysine-rich region), were dialyzed in PBS. In all experiments, the final concentration of HCALaβ2GPI was 1 mg/l.

IgG fraction was isolated from the serum of a patient with primary antiphospholipid syndrome. The IgG fraction contained high titers of anti-β2GPIs as determined by affinity purification on a 2 ml protein-G (protein that contains IgG binding domains) column (Pierce, Rockford, USA), using the protocol recommended by the manufacturer. The IgG fraction was equilibrated against PBS (pH 7.4) in a desalting column. This IgG fraction gave comparable results to whole serum in an anti-β2GPI ELISA (enzyme-linked immunosorbent assay, a serological assay in which bound antigen or antibody is detected by a linked enzyme, that converts a colorless substrate into a colored product) performed as described previously (Ambrožič et al., 2002). The levels of anti-β2GPIs in the IgG preparations were considered as medium range, amounting about a half of the anti-β2GPI levels in serum.

2.2. Giant phospholipid vesicles

GPVs were prepared at room temperature (23 °C) by the electroformation method (Angelova et al., 1992) with small modifications as described in (Tomšič et al., 2005). The synthetic lipids cardiolipin (1,1'2,2'-tetraoleoyl cardiolipin), POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). Weighted amounts of POPC, cardiolipin and cholesterol were all dissolved in a 2:1 chloroform/methanol (v/v) and thoroughly mixed. For charged cardiolipin vesicles, POPC, cholesterol and cardiolipin were mixed in the proportion

2:2:1 molar ratios. For neutral POPC vesicles, POPC and cholesterol were mixed in the proportion 4:1 (mol/mol). Cholesterol was added to POPC to increase the longevity of vesicles. Platinum electrodes were coated with lipid films by evaporation of 10 μl of solvent in a low vacuum for 2 h. The coated electrodes were placed in the electroformation chamber which was then filled with 3 ml of 0.2 M sucrose solution. An AC electric current with an amplitude of 5 V and a frequency of 10 Hz was applied to the electrodes for 2 h, which was followed by 2.5 V and 5 Hz for 15 min, 2.5 V and 2.5 Hz for 15 min and finally 1 V and 1 Hz for 15 min. The content was rinsed out of the electroformation chamber with 5 ml of 0.2 M glucose and stored in a plastic test tube. The vesicles were left for sedimentation under gravity for 1 day at 4 °C. Two hundred to four hundred microliters of the sediment was collected from the bottom of the tube and used for a series of experiments. Before placing the vesicles into the observation chamber, the sample was gently mixed.

2.3. Observation

Vesicles were observed by a Zeiss Axiovert 200 (Jena, Germany) inverted microscope with phase contrast optics (objective magnification 100×) and recorded by a Sony XC-77CE (Trnava, Slovakia) video camera. The solution containing vesicles was placed in an observation chamber made from cover glasses sealed with grease. The larger (bottom) cover glass was covered by two smaller (18 mm × 18 mm glasses), each having a small semicircular part removed at one side. Covering the bottom glass with two opposing smaller glasses thus formed a circular opening in the middle of the observation chamber. The circular opening enabled subsequent addition of PBS-dissolved protein. In all experiments the solution of vesicles (45 μl) was placed in the observation chamber. The solution containing the substance under investigation (5 μl) was added through the circular opening in the middle of the observation chamber. The osmolarity of the sample containing vesicles was 205 mosm/l (measured by a Knauer K7000 Vapor pressure osmometer, Berlin, Germany). During observation the chamber was mounted on a temperature-regulated microscope stage. In observing the coalescence of the population of vesicles the temperature was kept at 40 °C while the formation of the bud was promoted by increasing the temperature (Kas and Sackmann, 1991) above the room temperature until the bud of the required shape was formed (usually about 10 °C).

3. Results

β2GPI caused coalescence of cardiolipin-containing (Fig. 1A) as well as of POPC vesicles (Fig. 1B). Adhesion to the bottom of the observation chamber occurred simultaneously. Formation of sticky complexes was also observed in samples which contain both kinds of vesicles. This indicates that β2GPI mediates the interaction between the charged–charged, charged–neutral and neutral–neutral pairs of membranes.

Addition of HCALaβ2GPI dissolved in PBS caused coalescence of charged cardiolipin vesicles and their adhesion to the bottom of the observation chamber (Fig. 1C), while neutral POPC vesicles did not coalesce nor adhere to the bottom of the observation chamber (Fig. 1D). Addition of HCALaβ2GPI dissolved in PBS to the mixture of charged and neutral vesicles yielded two coexisting populations: one forming sticky complexes that adhered to the bottom of the observation chamber and the other consisting of separate fluctuating vesicles (not shown). When β2GPI and HCALaβ2GPI were well mixed and incubated for 10 min before they were added to the solution containing vesicles, we observed coalescence of cardiolipin-containing vesicles (Fig. 1E), but not of POPC vesicles (Fig. 1F).

Addition of the IgG fraction which contained anti-β2GPIs caused coalescence of the charged vesicles and their adhesion to the bottom of the observation chamber, while these effects were absent in neutral vesicles (Fig. 1G and H, respectively). The addition of the pre-incubated mixture of β2GPI and IgG caused coalescence of charged vesicles and of neutral vesicles (Fig. 1I); however, in neutral vesicles, the effect was very weak (Fig. 1J).

Figs. 2 and 3 illustrate the effect of phospate buffer saline and β2GPI on the budding membrane, respectively. The budding of the vesicle was induced by rising the temperature of the sample above room temperature. As a result of increasing the temperature, the bud elongated and appeared tube-like. When the tube was of a sufficient length the temperature was kept constant (typically 35 °C) and the substance under investigation was added into the observation chamber (β2GPI dissolved in PBS or PBS alone—for control). Fig. 2 shows the effect of PBS of higher osmolarity (283 mosm/l). The presence of PBS in the outer solution caused the bud to attain a bead-like shape (Fig. 2B). The bud detached from the mother vesicle (Fig. 2C) and decomposed into separate spherical vesicles (D and E), which were free to migrate away from the mother vesicle (F). When β2GPI was present in the solution, the bud (Fig. 3A–C) had coalesced with the mother vesicle before it could detach
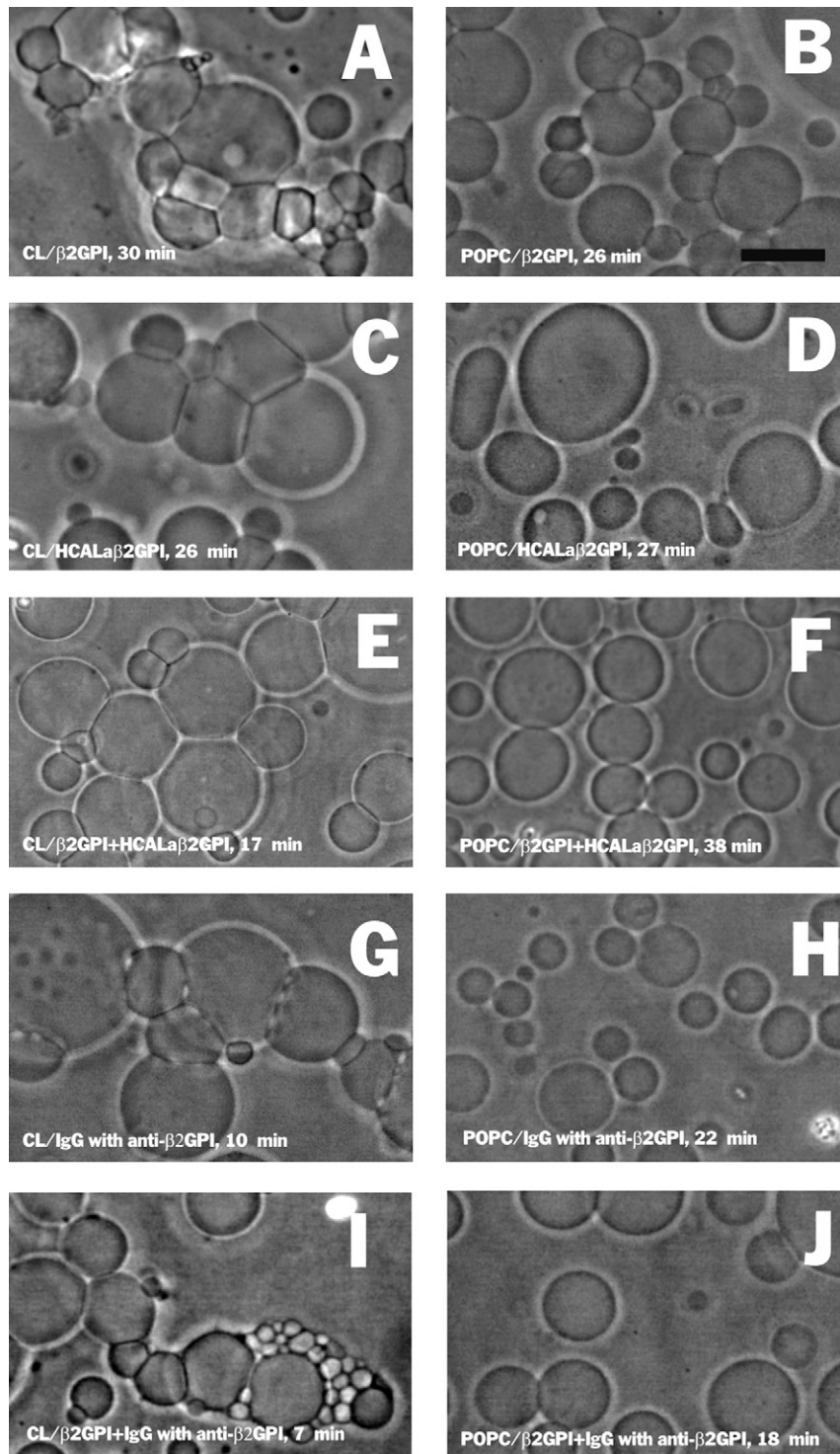
Fig. 1. Effect of β2GPI and anti-β2GPI antiphospholipid antibodies (anti-β2GPIs) on giant phospholipid vesicles. Left side (A, C, E, G, and I): negatively charged cardiolipin-containing vesicles, right side (B, D, F, H, and J): neutral POPC vesicles. Bar = 10 μm.
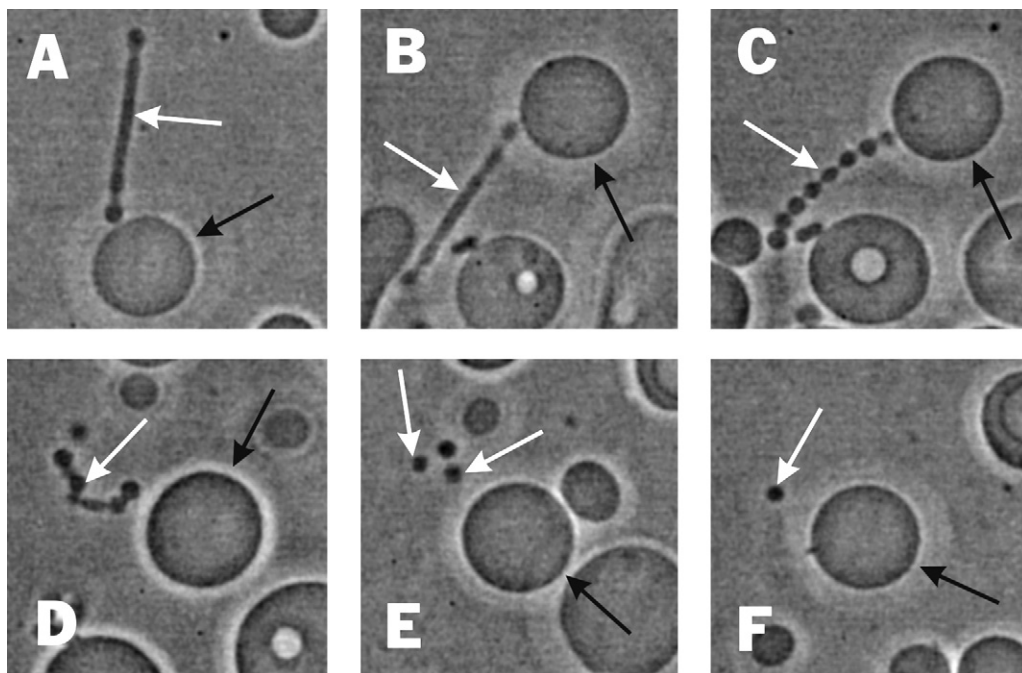
Fig. 2. (A–F) The effect of phosphate buffer saline (PBS) on a budding vesicle. The bead-like bud (marked by white arrows in B and C) detached from the mother vesicle (marked by black arrows) and disintegrated into spherical microvesicles (marked by white arrows in E and F) in the timescale of minutes. Bar = 10 μm.

from it (Fig. 3D–F). Fig. 3G shows a sticky complex formed by a vesicle with long, initially tubular protrusion. In contrast to the case presented in Fig. 2, the beads reunited with the mother vesicle.

4. Discussion

Our observations indicate that β2GPI and anti-β2GPIs importantly influence interactions between phospholipid assemblies.

It was found (McNeil et al., 1990; Galli et al., 1990; Matsuura et al., 1990) that antiphospholipid antibodies were directed to β2GPI complexed with cardiolipin. Based on the fact that highly purified (>95%) antiphospholipid antibodies do not bind to cardiolipin when assayed by a modified cardiolipin ELISA in which the blocking agent does not contain β2GPI, it was suggested that the presence of β2GPI is an absolute requirement for an antibody–phospholipid interaction (McNeil et al., 1990). But in contrast, we observed that the HCALaβ2GPI and the IgG fraction of a patient containing anti-β2GPI antibodies interact with cardiolipin-containing membrane, both in the presence and in the absence of β2GPI (Fig. 1C, E, G, and I).

Secondly, binding of β2GPI to negatively charged phospholipids has been considered to mediate the interaction between phospholipid assemblies and macrophages (Thiagarajan et al., 1999). The complex of anionic phospholipid vesicles and β2GPI was found to be a specific requirement for anionic phospholipids to be recognized by a putative cell surface receptor on macrophages. Nevertheless, we observed that β2GPI may also directly mediate attractive interactions between charged–charged (Fig. 1A), charged–neutral (not shown) and neutral–neutral phospholipid membranes (Fig. 1B).

Even when added alone, HCALaβ2GPI and patient's anti-β2GPIs were observed to act similarly to β2GPI in mediating the attractive interaction between negatively charged surfaces (Fig. 1C and G), but these antibodies alone or previously mixed with β2GPI failed to mediate the attractive interaction between neutral POPC membranes (Fig. 1D, H and F, J, respectively).

The electrical double layer is a model in which a charged surface is in contact with a solution containing counterions (ions charged oppositely to the charged surface) and coions (ions with the same charge as the charged surface). Counterions are attracted to the charged surface and accumulate near it while coions are repelled by the surface while the entropy opposes the ordering or the ions. A diffuse layer created by the ions is formed to shield the electric field created by the charged surface. The interaction between

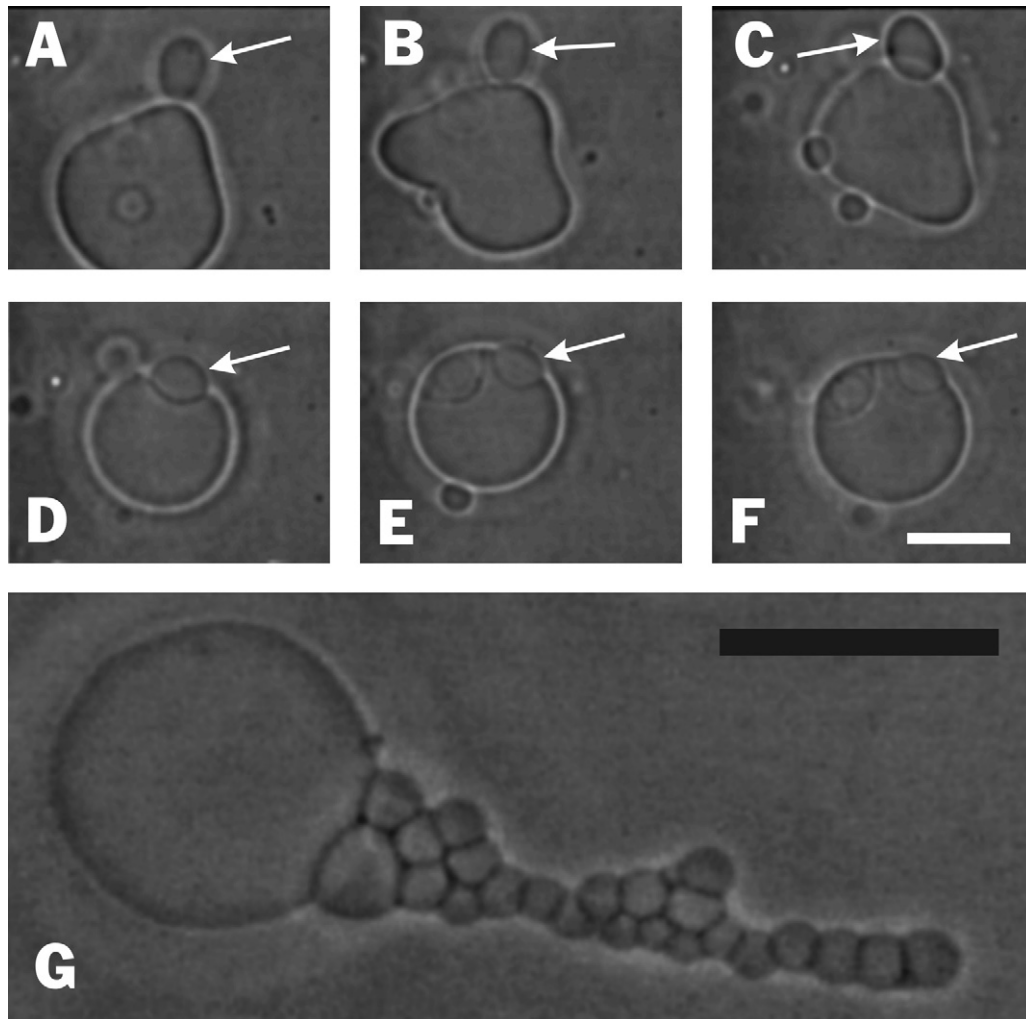Fig. 3. The effect of β2GPI dissolved in phosphate buffer saline (PBS) on a budding vesicle (A–F). The bud (marked by a white arrow) coalesced with the mother vesicle and remained attached to it. A sticky complex formed by coalescence of a bead-like protrusion and a mother vesicle (G). Bars = 10 μm.
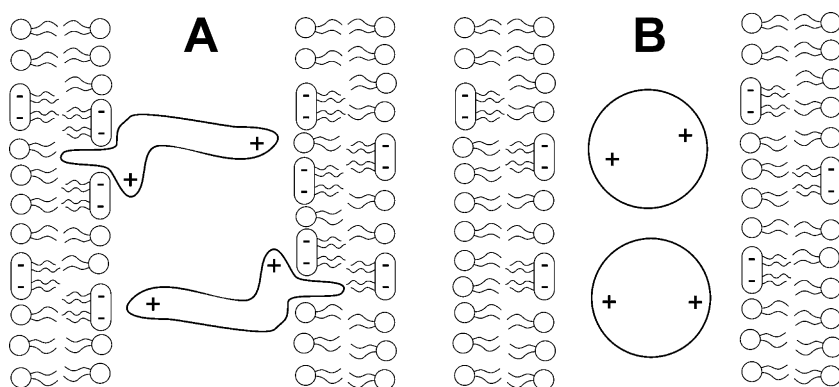

Fig. 4. Schemes of the β2GPI-mediated (A) and antibody-mediated (B) interactions between negatively charged phospholipid bilayers.

two negatively charged surfaces (formed by the negatively charged phospholipid headgroups) with a solution containing ions (including protein molecules, such as β2GPI and antibodies) between them can be described by interaction between two electrical double layers. The classical Poisson–Boltzmann theory (Verwey and Overbeek, 1948), which considers ions as dimensionless charges subject to the mean electrostatic field created by the charged surfaces and ions in the solution, yields repulsion between like-charged surfaces. On the contrary, we have observed attraction between membranes of negatively charged GPVs and adhesion between them. The affinity of β2GPI for apoptotic membrane blebs that contain phospholipids with negatively charged headgroups can be explained by their opposite charges, i.e. some β2GPI domains are highly positively charged (Kertesz et al., 1995; Bouma et al., 1999). However, it was found that besides electrostatic interactions β2GPI also binds to phospholipid layers by hydrophobic interaction (Wang et al., 1998). Therefore, a β2GPI molecule can intercalate in the membrane of the GPV with its hydrophobic part and is simultaneously attracted to the membrane of another GPV with its poly-lysine segment on the I-st domain thereby forming a bridge between the membranes of the vesicles (Fig. 4A). Such configuration is favorable with respect to the electostatic energy of the electric double layer. The antibodies, being composed of two heavy and two light polypeptide chains, form a dimeric structure with a particular internal distribution of charge. Within a simple model such structure can be represented by a dimeric ion consisting of two equal point charges at a fixed distance apart. Orientational ordering of dimeric ions in the gradient of the

local electric field (Fig. 4B) gives rise to an attractive force between the two electric double layers (Bohinc et al., 2004). The above mechanisms are suggested to prevail in the observed attractive interactions between charged membranes involving β2GPI and anti-β2GPIs (Fig. 1A, C, E, G, and I), although there may be other contributions to the interaction, such as van der Waals attraction, suppression of fluctuations (Helfrich, 1995) and water ordering near the interfaces (Israelachvili and Wennerstrom, 1996).

Microvesicles that are shed off cells represent an important system of transport of matter and information within the body (Greenwalt, 2006; Ratajczak et al., 2006). They can be released by all cell types upon activation or apoptosis (Distler et al., 2005), moreover, they may play an important role in vascular disease (Diamant et al., 2004) and in communication of tumor cells with macrophages (Baj-Krzyworzeka et al., 2005). There may be different mechanisms that promote budding, such as redistribution of membrane constituents (Hagerstrand et al., 2006), increase of the area of the outer layer with respect to the area of the inner layer (Sheetz and Singer, 1976; Lipowsky, 1991) and increase of the temperature (Kas and Sackmann, 1991). In this work, we have induced the budding by increasing the temperature, however, the effect of the adhesion mediators on the budding vesicles can be considered as general. By causing adhesion of buds to the mother cell (Fig. 3), the presence of certain serum proteins could suppress the release of cell membrane exovesicles into the circulation thereby causing a decrease of the number of circulating microvesicles (see also the scheme for the mechanism depicted in Fig. 5). The proposed hypohesis
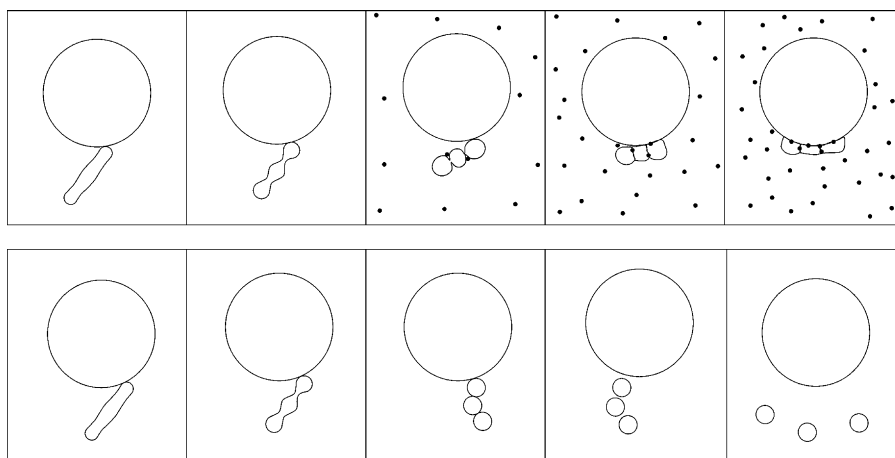

Fig. 5. Scheme of the budding and vesiculation of the membrane in the presence (upper) and in the absence (lower) of adhesion mediators. If adhesion mediators are present, the buds adhere to the membrane, while if adhesion mediators are absent, the buds pinch off the mother membrane and become microexovesicles.

is supported by the facts that a decrease of the level of plasma β2GPI (Brighton et al., 1996) and an increase of the level of prothrombogenic microvesicles (Morel et al., 2004; Dignat-George et al., 2004) was observed in patients with disseminated intravascular coagulation.

However, as this mechanism is of general, non-specific nature, it could involve many kinds of plasma constituents and thus, further laboratory and clinical studies are necessary to assess the extent and possibilities of the proposed mechanism. Budding and vesiculation observed in GPVs are expected to take place also in cells. Microvesiculation of erythrocyte membrane can be induced in vitro by incubation with detergent in the presence of different plasma constituents, such as β2GPI, anti-β2GPI antibodies and β2GPI–anti-β2GPI complexes. A reduced amount of obtained microvesicles (which can be measured by flow cytometry) with respect to the control (incubated with detergent only) would point to anticoagulant effect of the substance under investigation. In clinical studies involving a population of blood donors, it would be of interest to determine the strength of the mediating effect of different plasma constituents on the interaction between GPVs and test for possible correlations with the amount of "native" microvesicles isolated from plasma. The quantification of the strength of the mediating effect (for example by assessing the angles of contact between adhered GPVs (Evans, 1995)) represents a challenge as the population of GPVs is heterogeneous with respect to size, shape and relative proportions of constituents. Also, in order to better understand the mechanisms underlying the mediated interaction between membranes, it is of importance to study the effects of the concentration of the studied mediators and of the temperature on the strength of adhesion between GPVs.

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