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Protein-Dependent Lipid Lateral Phase Separation as a Mechanism of Human Erythrocyte Ghost Resealing

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The hypothesis of a correlation between a 10°-20°C lipid phase transition and the resealing process of human erythrocyte membrane has been investigated. The conditions required to reseal human erythrocyte ghosts have been studied by measuring the amount of fluorescein-labeled dextran (FD) that is trapped into the membrane.

Temperature per se was sufficient to induce membrane resealing: (1) at 5 mM sodium phosphate, pH 7.8 (5P8), resealing began at 12°C; (2) at salt concentrations above 8 mM sodium phosphate, it occurred at lower temperature; and (3) in isotonic saline was detected just above 5°C.

The removal of peripheral membrane proteins from unsealed membranes by chymotrypsin at 0°C in 5P8 was followed by membrane resealing.

This seems to imply that the presence of proteins is necessary to maintain the membrane unsealed. Protein-induced lateral phase separation of lipids may be a reasonable mechanism for the observed phenomena. In fact, the permeability of phosphatidylserine-phosphatidylcholine mixed liposomes to FD is modified by lipid lateral phase separation induced by pH or poly-L-lysine.

Electron spin resonance studies of membrane fluidity by a spin labeled stearic acid showed a fluidity break around 11°C, which may be due to a gel-liquid phase transition. Fluidity changes are abolished by chymotrypsin treatment.

It is suggested that a lateral phase separation is responsible for the permeability of open ghosts to FD. Accordingly, disruption of phase separation apparently produces membrane reconstitution. In this respect peripheral proteins and particularly the spectrin-actin network, may play a major role in membrane resealing.

Key words: ghost resealing, fluorescein-labeled dextran, lipid lateral phase separation, EPR studies, cytoskeletal protein-lipid interactions

Abbreviations used: FD, fluoresceinylthiocarbamoyl-dextran (the number following FD indicates the approximate molecular weight in thousands); 16NS, 2-(14-carboxy-tetradecyl)-2-ethyl-4, 4-dimethyl-3-oxazoli-dinyloxyl; EPR, electron paramagnetic resonance; 5P8, 5 mM Na phosphate, pH 7.8; RBC, red blood cell.

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An increasing body of data supports the hypothesis that membrane components are not simply mixed in a random fashion, but that they interact in such a way as to segregate or pool into specialized regions. One of the possible mechanisms for generating a mosaic distribution of membrane components is a phase separation due to the gel-liquid transition of lipid acyl chains. From this point of view, temperature-dependent membrane processes can, in some cases, be accounted for by the thermotropic properties of membrane lipids.

After hypotonic hemolysis, the erythrocyte membrane can regain the low cation permeability of intact erythrocytes if the temperature and buffer osmolarity is raised up to physiological values [1]. This process known as "resealing" depends not only on a given membrane treatment necessary for its reconstitution but also on the conditions at hemolysis [2]. The membrane components involved in ghost reconstitution are not exactly known but both lipids and proteins seem to be important. In fact, a role for lipids in the fluid state is suggested by the temperature dependence of the process, and the involvement of proteins is deduced by the requirement of an intact spectrin network for resealing [3].

Several authors using different physicochemical methods [4–10] reported a gel–liquid phase transition in the region of 10°–20°C for either intact erythrocyte [4,5,8] or ghost membranes [5,7,9,10], or various derivatives thereof [7]. A phase transition in this range of temperature is surprising: in fact, since in the erythrocyte membrane the molar ratio of cholesterol to phospholipids is about 0.8, a random distribution of the sterol would abolish any cooperative phase transition. However, there are hints that such a random distribution of cholesterol may not occur [11–13]. In addition, the reported gel–liquid phase transition in this range of temperature implies an heterogeneous lipid distribution.

In the work reported in this paper we tested the hypothesis of a correlation between the 10°–20°C phase transition and the resealing process of ghosts after hypotonic hemolysis. The data show that the resealing process depends on temperature, ionic strength, and extrinsic membrane proteins. Spin label studies show that a gel–liquid phase transition may be taking place in the same temperature range of the resealing process. Proteolysis of extrinsic membrane proteins produces both ghost resealing and the disappearance of gel–liquid transition. We suggest that the triggering for resealing is the disorganization of a protein–lipid lateral phase separation present in open ghosts at low temperature and low salt concentration.

MATERIALS AND METHODS

Fluorescein-labeled dextrans (FD) were obtained from Pharmacia (Upsala, Sweden). Unless otherwise noted the molecular weight of FD used in this study was 39,000 daltons. Egg phosphatidyl-choline and brain phosphatidylserine were obtained from Calbiochem (San Diego, California); Chymotrypsin A_4, phospholipase D (cabbage) and phospholipase A_2 (hog pancreas) from Boehringer (Mannheim, GmbH); trypsin from Fluka (Buchs, Switzerland); and poly-L-lysine from Miles (Milano, Italy). 2-(14-carboxy-tetradecyl)-2-ethyl-4, 4-dimethyl-3-oxazolidinyloxyl (16 NS) was obtained from Syva (Palo Alto, California).

Ghost Preparation and Resealing Process

Fresh human erythrocytes were washed three times by centrifugation in phosphate buffered saline (5 mM sodium phosphate, 0.15 M NaCl, pH 7.8). After each

centrifugation the buffy coat was removed by aspiration. To minimize individual variability, all the experiments were performed by using a pool of membranes from 5–12 donors.

Erythrocytes were lysed in 5 mM sodium phosphate, pH 7.8 (5P8), according to Steck and Kant [14], except that a cell:buffer ratio of 1:10 was chosen to reduce spectrin loss [15]. The temperature was maintained around 0°C during all the procedures and ghosts were collected by a 10-min centrifugation at 47,800 g. Usually three washes with the lysing buffer were sufficient to obtain white ghosts with a protein concentration of 3–4 mg/ml.

To study resealing, one volume of a 50 mg/ml solution of FD in 5P8 was rapidly mixed at 0°C with nine volumes of ghosts in the same buffer. After pre-equilibration for 5 min an equal volume of cold buffer with 5 mg/ml FD and the desired ionic composition was added. The pre-equilibration step is necessary since the internal volume of ghosts does not immediately equilibrate with added solutes [3]. To start resealing, the sample was transferred at the chosen temperature. An incubation for 20 min allowed a maximum FD trapping. At the end of the incubation time, samples were diluted 1:10 with a cold buffer with the same ionic composition but without FD, and centrifuged at 15,000 g for 5 min. Finally ghosts were washed three times. It is to be emphasized that washings of unbound FD from ghosts were performed in the same buffer used during the incubation. In this manner membranes did not undergo osmolarity of pH shock that could modify polymer trapping.

Membranes were resuspended and observed with a Leitz Orthoplan fluorescence photomicroscope (Ernst Leitz, Wetzlar, West Germany). Determination of bound FD was performed by dissolving pellets in 10% sodiumdodecyl sulphate at room temperature and fluorescence was measured with an Aminco-Bowman spectrofluorometer (American Instruments Co., Silver Spring, Maryland). To study the releasing of FD trapped into ghosts, membranes were diluted ten times with a buffer with the desired ionic composition and incubated for 1 hr at 0°C. After 5 min centrifugation at 15,000 g the pH of supernatants was corrected to pH 7.5 and fluorescence measured. All of the trapped FD was obtained by dissolving the same membrane amount used in releasing studies in 10% sodium-dodecyl sulphate.

Enzymatic Treatments

Phospholipase A_2 and phospholipase D treatments of intact erythrocytes was performed according to Haest and Deuticke [16]. To increase phospholipase activity, erythrocytes were preincubated with 5 mM tetrathionate at 37°C for 3 hr. Proteolytic digestions of intact erythrocytes and unsealed ghosts were performed with 1 mg/ml chymotrypsin or trypsin at 0°C for 1 hr [17].

To control the effectiveness of protease treatments, erythrocyte membranes were submitted to SDS gel electrophoresis. To evidence the effects of proteolytic treatments on externally exposed proteins, erythrocytes were labeled with fluorescein-iso-thiocyanate according to Fowler and Branton [18]. At the end of proteolytic treatments, phenylmethyl sulfonyl fluoride (0.1 mM) was included in the phosphate buffers. Polymer trapping into ghosts during chymotrypsin treatment was performed by adding FD (1 mg/ml) during proteolysis.

Multilamellar Liposomes

To prepare multilamellar liposomes lipids solutions were evaporated under N_2 and stored for at least 1 hr under high vacuum. FD releasing from liposomes was

studied by adding the polymer (1 mg/ml) in the swelling buffer (5 mM sodium phosphate, pH 7.8, 0.05 mM EDTA). Swelling was obtained by vortexing lipid films at 37°C. To increase the size of multilamellar liposomes the samples were stored overnight at 4°C at a lipid concentration of 20 mg/ml. Liposomes containing trapped FD were washed three times and 1 mg of lipids was incubated at room temperature with 1 ml of buffer with the desired pH or with a known concentration of poly-L-lysine. After 1 hr incubation at room temperature, liposomes were sedimented by 5-min centrifugation at 15,000 g, and the fluorescence of supernatants measured. Acidic and alkaline pH were adjusted to pH 7.5 before fluorescence determination.

EPR Measurements

The 16-doxyl stearic acid spin probe (16 NS) was dissolved in absolute ethanol at a concentration of 10 mM and pipetted into test tubes; the ethanol was evaporated under a stream of nitrogen.

Membrane incorporation of the label was carried out at 0°C for 30 min for unsealed ghosts and at 37°C for 1 hr for intact erythrocytes. In both cases the label concentration was 10 μg of stearic acid spin label per mg of membrane proteins. In addition, to minimize possible differences between samples, the following precautions were used: (i) Erythrocytes were prepared from a mixture of 8–10 donors bled within 12–24 hr. (ii) The comparison between intact erythrocytes, unsealed ghosts, and chymotrypsin-digested ghosts was carried out with the same red cell sample and on the same day. (iii) To obtain a spin label distribution as similar as possible between unsealed and chymotrypsin-digested ghosts, the label incorporation was performed before the proteolytic treatment. The EPR spectra were recorded on a Varian E-4 spectrometer (Milan, Italy) equipped with a digital thermometer set above the cavity. At each temperature four spectra were recorded and the average value of peak heights (see Results) used for calculations.

RESULTS

FD Interaction With Erythrocyte Membrane as a Marker for Ghost Resealing

It has been suggested [19,20] that (i) the membrane components involved in the binding between neutral polysaccharides, such as dextrans, and the erythrocyte membrane are lipids, and (ii) the binding consists of polymer adsorption at the lipid–water interface. Data in Table I strengthen this hypothesis by showing that FD binding to intact erythrocytes can be reduced or abolished by phospholipase treatment of RBC. This effect is due to the enzymatic activity itself and, in fact, it was noticeable only after pretreatment of erythrocytes with 5 mM tetrathionate (Table I). On the other hand it is known [16] that RBC membranes not subjected to this treatment are marginally or not at all digested by phospholipases. RBC surface proteins seem to play an inhibitory role in FD adsorption, as extensive proteolytic treatments increase polymer binding (Table I). This effect could be due both to the unmasking of lipidic areas and to surface charge reductions [21] as a consequence of glycoproteins removal.

TABLE I. Effect of Enzymatic Treatments of Human Erythrocyte on FD Adsorption

Enzyme	RBC pretreatment	Fluorescence[a]
Phospholipase A_2	–	+ +
Phospholipase A_2	Tetrathionate 5 mM, 3 hours, 37°C	±
Phospholipase D	–	+ +
Phospholipase D	Tetrathionate 5 mM, 3 hours, 37°C	–
Trypsin	–	+ + +
Trypsin + Chymotrypsin	–	+ + +
–	–	+ +
–	Tetrathionate 5 mM, 3 hours, 37°C	+ +

[a]Arbitrary units (– no fluorescence, ± hardly detectable fluorescence, + detectable fluorescence, + + evident fluorescence, + + + intense fluorescence). Final FD concentration 50 mg/ml. RBC 1 × 10^8 cells/ml.

The choice of FD as a tool to study the resealing process is based on the following considerations: (i) negligible effect on solution osmolarity, (ii) chemical and enzymatic stability [22], (iii) potential for control of membrane morphology by fluorescence microscopy, (iv) detectability also of small amounts of bound polymer, and (v) a weak perturbation of lipid thermotropic properties and lipid packing in model membranes of synthetic lecithins [23,24]. In conclusion, when ghosts are permeable to FD, the amount of FD found with membranes after washings is due only to polymer suface adsorption. Conversely, when ghosts reseal, the amount of FD found with membranes is increased by polymer trapping inside the cell.

Effects of Temperature and Ionic Strength on Ghost Resealing

In Figure 1 the effects of temperature on the fluorescence pattern of FD after incubation with RBC ghosts are shown. At 0°C most ghosts are open and only a faint membrane fluorescence is visible for ghosts incubated both in the lysing (heretofore designated 5P8, see also Methods) or in saline buffer (Fig. 1a,b). In the same buffers at 37°C membranes reseal as shown by the presence of intensely fluorescent ghosts (Fig. 1c,d). In 5P8 the morphology of the ghosts is more heterogeneous and small vesicles are present (Fig. 1c).

The amounts (expressed in arbitrary units) of fluorescent polymer remaining trapped into RBC ghosts at different temperatures are shown in Figure 2. The upper curve represents FD remaining trapped into ghosts incubated in phosphate-buffered saline, pH 7.8. It is evident that the amounts of FD abruptly increase at temperature values above 5°C whereby fluorescence microscopy observation shows polymer trapping due to the reconstitution of the membrane impermeability to polymer. Above 30°C trapping of FD is maximal and a 100% coincidence between ghosts observed by phase contrast and fluorescent structures was found. The above saline-treated membranes subjected to gradient separation according to Kanda et al [25] were homogeneously recovered from the gradient top; thus confirming their complete resealing. If ghost incubation is performed in 5P8 the temperature at which resealing begins rises to 12°C. At 41°C in 5P8 the amount of FD trapped does not reach plateau values but is only one-half that observed with saline buffer (Fig. 2). Under these conditions not all the ghosts are fluorescent. This could be due to a

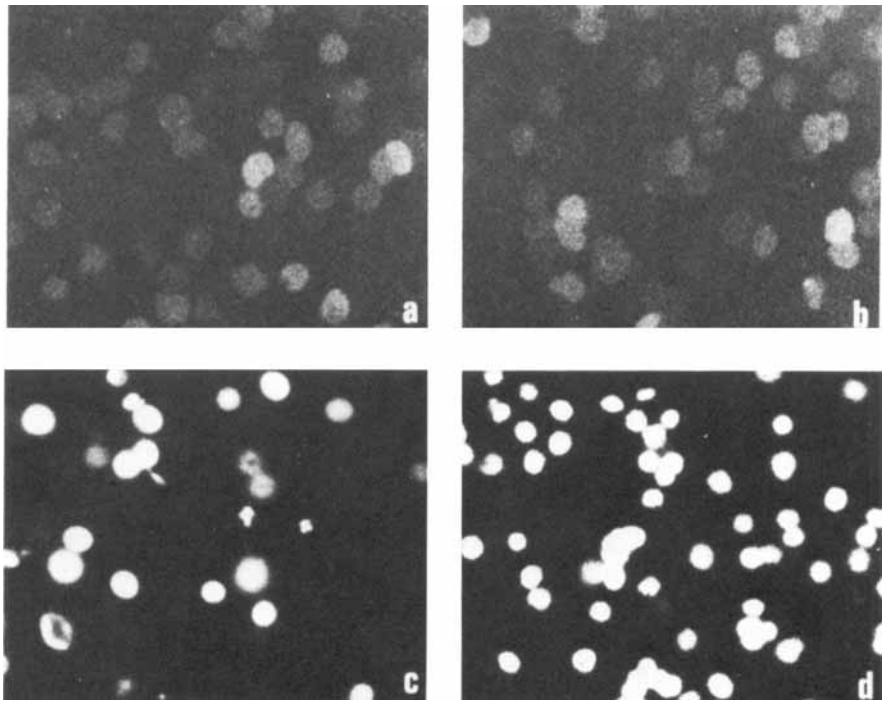

Fig. 1. Fluorescence photomicrographs of RBC ghosts containing trapped FD. (a–c) Ghosts after incubation in 5P8 at 0°C (a) or at 37°C (c). (b–d) Ghosts after incubation in phosphate buffered saline at 0°C (b) or at 37°C (d). Final concentrations used during the incubations: FD 15 mg/ml, RBC ghosts 4 mg/ml. × 375.

partially reversible resealing. Perhaps some trapped FD is released during membrane washings. It is tempting to assume that the presence of salts during the resealing process results first in ghosts that can stably trap the polymer into the cell (possibly by hindering spectrin loss at low ionic strength [3,15]), and second in the lowering of the resealing temperature. At sodium phosphate concentration above 8 mM a rapid increase in the amount of FD trapped at 12°C has been observed, thus suggesting that at these salt concentrations ghosts reseal below 12°C.

To rule out the possibility that resealing at low ionic strength is a phenomenon somehow dependent upon the presence of FD, ghosts heated in the absence of the polymer in 5P8 were then incubated with FD at different temperatures. Ghosts heating at 20° or 41°C for 20 min in 5P8 produce lower polymer trapping during the subsequent incubation step (Fig. 2; dotted lines). These results indicate that preheating of ghosts per se is sufficient to partially reconstitute membrane permeability barrier, thus hindering FD trapping.

It is interesting to note that ghosts resealed by saline are subjected to the same lysis conditions as intact RBC. In saline buffer at 0°C resealed ghosts containing trapped FD do not lyse after inclubation for 2 hr, but if the external buffer is replaced with 5P8 a 90% polymer releasing is observed.

Since resealing is induced by temperature, also in the absence of FD (Fig. 2, dotted lines), the polymer itself does not seem to play a role. This is strengthened by

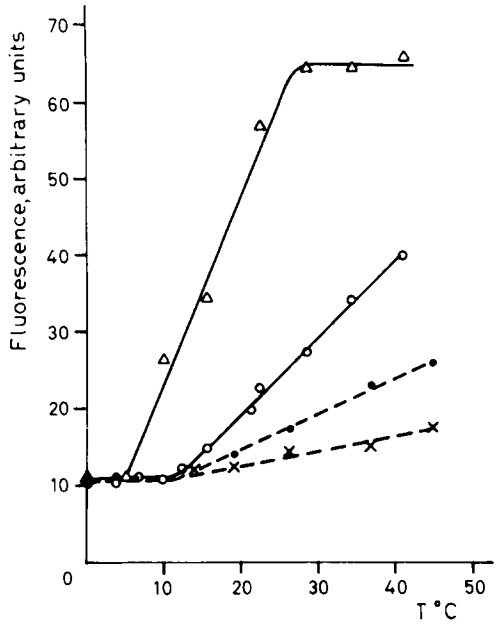

Fig. 2. Trapping of FD into ghosts as a function of temperature . Ghosts incubated in phosphate buffered saline (△—△) or in 5P8 (○—○). Dotted lines: effect of 20-min preincubation in 5P8 at 20°C (●--●) and at 41°C (× --×). Final concentrations used during the incubations: FD 5 mg/ml, RBC ghosts 4 mg/ml. Fluorescence: 484 nm excitation; 525 nm emission.

data in Figure 3 where the amount of trapped polymer is plotted against the molecular weight of various FD. If FD were to enhance ghost resealing, a nonlinear relationship of polymer trapping to its molecular weight would be expected. Both at 0°C and 37°C, however, a linear relationship with identical slopes was found (Fig. 3). The observation that polymer adsorption to the membrane surface at 0°C raises along with increasing FD molecular weights is in accordance with data previously reported with intact RBC and model membranes [19,23,26,27].

Effects of Proteolytic Treatment on Ghost Resealing

Since proteins may play a role in the resealing process, the effects of proteolytic treatment on RBC membrane have been investigated. To avoid temperature-induced resealing, proteolytic digestion of unsealed ghosts had to be carried out at low temperature. As reported by Weinstein et al. [17], chymotrypsin treatment of unsealed ghosts at 0°C for 1 hr produces extensive digestion of the major membrane proteins (no bands were detectable on SDS gel electrophoresis) but leaves the so-called intramembrane particles morphologically intact. Thus chymotrypsin digested ghosts are a good model to investigate protein-lipid interactions in RBC membrane since, after proteolysis, proteins experiencing polar interactions with lipids are preferentially removed.

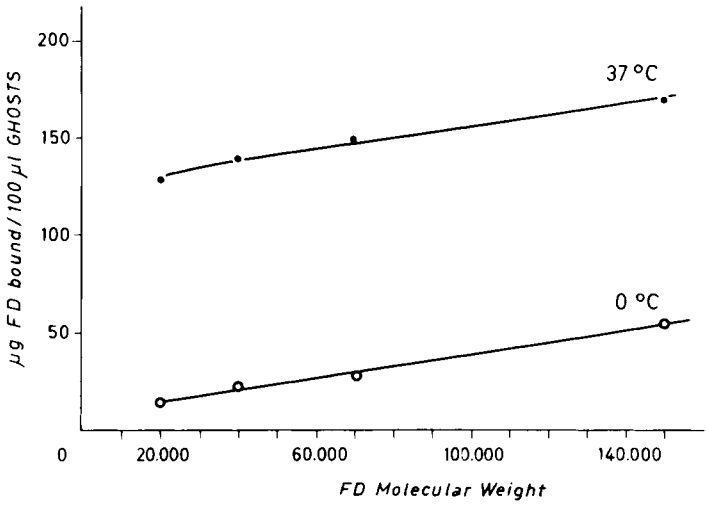

Fig. 3. Molecular weight dependence on the amount of FD trapped into RBC ghosts in 5P8 at 0°C and 37°C. FD and RBC concentrations as in Figure 2. FD molecular weights are manufacturer's data.

As reported in Figure 4, the chymotrypsin treatment of open ghosts inhibits the temperature-dependent trapping of FD. This effect can be due either to the formation of sealed membranes as a consequence of proteolysis, or to the inability of chymotrypsin-digested ghosts to reseal during heating. To distinguish between these two possibilities the polymer was added during the proteolytic digestion. Sealed round membranes containing trapped FD were observed, thus confirming the protease-induced resealing hypothesis (Fig. 5). The amount of FD trapped into these chymotrypsin-digested ghosts at 0°C was as much as that obtained with ghosts resealed by saline at 37°C, thus suggesting that the resealing is complete.

If the same proteolytic treatment is performed on intact RBC membranes, only the external part of intrinsic proteins is digested, in accordance with previously reported data [28,29], without any modification of cytoskeletal proteins. After hemolysis these membranes resealed at a temperature slightly lower than untreated membranes in 5P8 (9°C and 12°C, respectively) and contained increased amount of trapped polymer. The resealing obtained with externally digested RBC, although higher than that obtained with untreated ghosts, was not complete (Fig. 4). Therefore external proteins are less crucial for membrane resealing.

The increased amount of FD bound to protease-treated intact RBC membranes (Fig. 4, △) is due to membrane adsorption rather than to partial resealing (fluorescence microscopy, data not shown).

The strong effect of chymotrypsin on open ghosts suggest that internal proteins experiencing polar interactions with the bilayer may be important in maintaining the membrane unsealed.

To identify the ionizable groups of lipids that may be involved in the interaction with proteins, we have investigated the pH effect on membranes resealed by chymotrypsin treatment. In these experiments (Fig. 6) FD was incorporated into ghosts during the proteolytic treatment and the polymer released from vesicles was measured for each external pH change. Below pH 5 membranes became leaky and

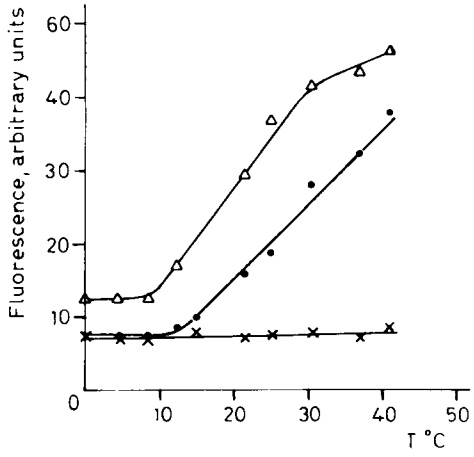

Fig. 4. Effect of chymotrypsin treatment of RBC on FD trapping. RBC were digested with chymotrypsin (1 hr, 0°C, 5P8) before (△) or after (×) the hemolysis. Undigested unsealed ghosts (●). FD and RBC ghost concentrations as in Figure 2.

at pH 2 polymer releasing was maxiumum after 1-hr incubation . By increasing the incubation time to 12 hr only an additional 15% of FD releasing was obtained at each pH tested (Fig. 6, dotted line). The acidic groups involved at low pH in the lysis of these protein-depleted membranes may be phosphatidylserine. Carboxylic group of phosphatidylserine shows, in fact, in model membranes a pK around 4 [33, 34].

Multilamellar Liposomes

Permeability to macromolecules observed in open ghosts before resealing is likely to be due to an interruption of the lipid barrier resulting in holes through which macromolecules can freely move. Thus lipid properties that are able to modify the bilayer permeability to macromolecules could, by themselves, be responsible for membrane lysis and resealing. Where this hypothesis is correct, resealing phenomena should take place also in model membranes (i.e., liposomes). The phase transition from bilayer to the hexagonal (H_{II}) phase is known to produce the disappearance of the permeability barrier of liposomes [32,33]. Nevertheless, the ability of the phospholipids to assume the H_{II} phase shows an opposite temperature dependence when compared to ghost resealing since the H_{II} phase is induced by increasing the temperature [34,35]. In addition, in our membrane preparations, a ^{31}P-NMR lineshape indicative of non-bilayer structures was not observed. Thus, this phase transition seems not involved in RBC membrane resealing [Minetti and Viti, unpublished results].

Also the gel–liquid phase transition and the lipid lateral phase separation can affect the permeability of liposomes [23,36]. However, since lipids extracted from the RBC membrane do not show any cooperative gel–liquid transition [7,37–39], only the lipid lateral phase separation has been looked into.

To prove whether lipid lateral phase separation modifies membrane permeability to FD, multilamellar liposomes from a 1:1 mixture of brain phosphatidylserine and egg lecithin were chosen as a model system. In these liposomes no tempera-

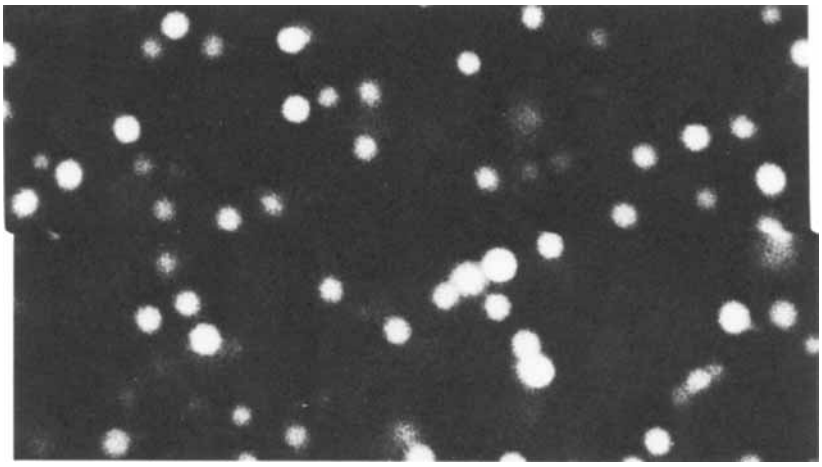

Fig. 5. Vesicles obtained by chymotrypsin treatment of RBC ghosts. Polymer was added during chymotrypsin treatment at 0°C and in 5P8. Polymer trapping shows that vesicles are sealed. × 625.

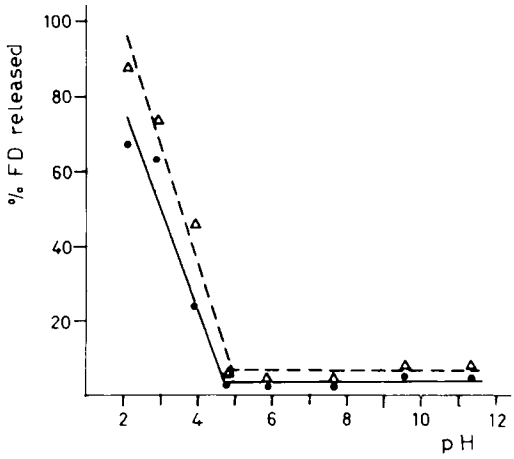

Fig. 6. pH effect on the releasing of FD from chymotrypsin digested ghosts. FD released was measured at each pH after 1 hr (●) or 12 hr (△) incubation. The amount of FD released was expressed as percentage of total FD trapped.

ture-dependent cooperative phase transition in the range between 5°–40°C takes place (brain phosphatidylserine alone shows a broad phase transition centered around 10°C [40]). Therefore, at room temperature, these membranes allow study of ionotropic lateral phase separation, which can be modulated in several ways [30,41–44].

Phosphatidylserine, for example, forms solid aggregates and separates from fluid phosphatidylcholine at low pH [30,45]. FD was incorporated into liposomes during the swelling time and polymer releasing from multilamellar liposomes was measured at various pH. In Figure 7, the percentage of FD release from phosphatidylserine-phosphatidylcholine liposomes after 1 hr of incubation at different pH is

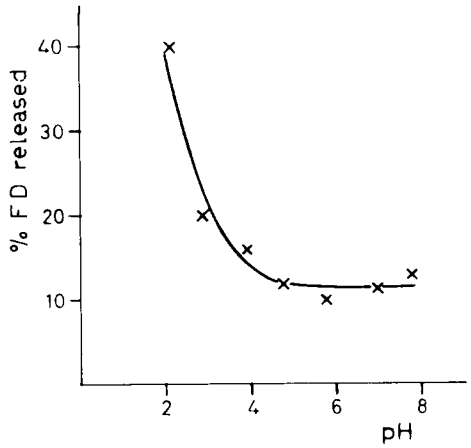

Fig. 7. pH effect on the releasing of FD trapped into phosphatidylserine-phosphatidylcholine multilamellar liposomes. FD and lipid concentrations, during liposome swelling, were 1 mg/ml and 20 mg/ml, respectively. The amount of FD released expressed as percentage of total FD trapped.

reported. When pH was reduced below the pK of phosphatidylserine carboxyl group, FD releasing was observed and was maximum at pH 2. Phosphatidylserine lateral phase separation can be induced also by poly-L-lysine, which appears to be a reasonable model for some extrinsic membrane proteins. In fact, it is known that this polypeptide experiences strong electrostatic interactions with negatively charged phospholipids [46], and in mixed systems it can induce lateral phase separation [47]. Data in Figure 8 show that in our mixed phosphatidylserine-phosphatidylcholine system, it is possible to produce polymer releasing, at room temperature, by increasing poly-L-lysine concentration. These results indicate that also electrostatic interactions between charged lipids and proteins are sufficient to induce FD releasing from membranes.

The broad dependence of FD release on poly-L-lysine concentration (Fig. 8) is due, probably, to the multilamellar structure of these model membranes. In fact, the releasing of FD, trapped within liposome layers, takes place gradually with phase separation and protein penetration into the underlying membrane. As a consequence, the lipid surface available to protein binding increases along with poly-L-lysine concentration. Nevertheless, the minimal protein concentration necessary to produce 50% FD release is on the order of a few micrograms per milliliter.

EPR Studies

The 16-doxyl stearic acid (16 NS) spin probe, used in this work, explores the hydrophobic core of the membrane and has been successfully utilized on both artificial and natural membranes [8,48–50]. This label is completely incorporated into membranes [49] and no free label is detectable in the supernatant.

The spectra of RBC ghosts incubated with the 16 NS are typical of a probe experiencing anisotropic motions into a biological membrane with a correlation time of the order of 10^{-9} sec. (Fig. 9A). The spectra of 16 NS in RBC can be interpreted by measuring the amplitude ratio of lines h_0 and h_1; h_0 being the peak height of the midfield line and h_{-1} the peak height of the highfield line (as reported in Fig. 9A).

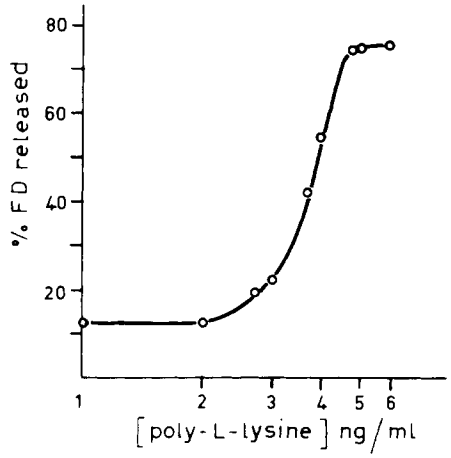

Fig. 8. Effect of poly-L-lysine on FD releasing from phosphatidylserine-phosphatidylcholine multilamellar liposomes. FD and lipids concentrations as in Figure 7. The log of poly-L-lysine concentration is plotted against the percentage of FD released.

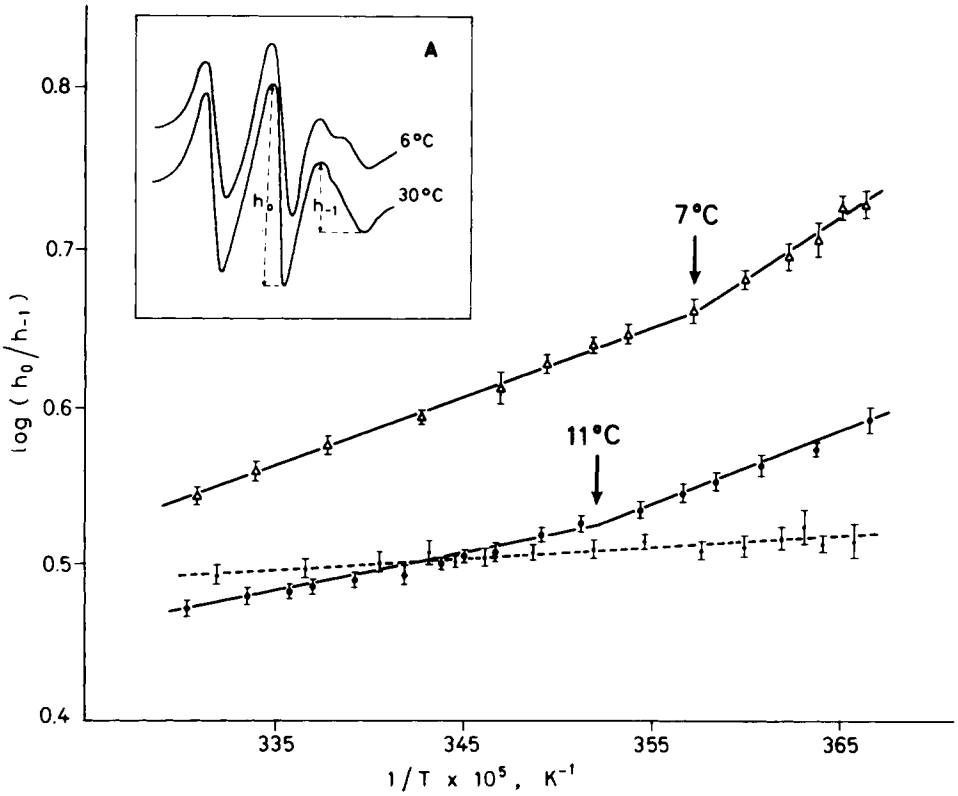

Fig. 9. The plot of log (h_0/h_{-1}) versus 1/T for 16 NS labeled RBC membranes. Intact RBC (△); unsealed ghosts (○), and chymotrypsin-digested ghosts (●). Values are given as means ± SEM. Panel A: Typical EPR spectra of unsealed RBC ghosts at 6° and 30°C. The parameters used for the plot are indicated.

This ratio may be used as an empirical relative measurement of fluidity [8,48]. The plot of log (h_0/h_{-1}) against the reciprocal of the temperature for intact RBC and for both unsealed and chymotrypsin-digested ghosts is reported in Figure 9. This plot shows a clear inflection point at 7°C and 11°C for intact erythrocytes and unsealed ghosts, respectively; whereas no break is observable for chymotrypsin-digested ghosts. Thus the presence of proteins seems to be important both in membrane resealing and in the fluidity change experienced by the spin label. Moreover, resealing of ghosts induced by salt and temperature (0.15 M NaCl, 37°C), does not produce the disappearance of the fluidity break (data not shown).

DISCUSSION

Data reported show that FD can be used to monitor membrane permeability changes to macromolecules due to the resealing process, which depends on temperature, ionic strength, and membrane proteins.

Temperature seems to be the most important factor. As observed also by Bodeman and Passow [51], the ghosts are unsealed at 0°C and no membrane reconstitution is observed at this temperature, even in presence of saline buffer (Fig. 1b).

According to Johnson and Kirkwood [3], at low ionic strength temperature is not sufficient to induce ghosts resealing, whereas, in our hands, as well as in those of Kanda et al. [25], it is possible to produce 50% resealing in 5P8 buffer by increasing the temperature to 41°C. The discrepancy between our data and those of Johnson and Kirkwood [3] probably lies in differences in ghost preparation and/or in the macromolecule used to monitor the resealing.

As previously suggested by Lepke and Passow [2], the effects of salts in resealing suggest a role of ionizable groups in membrane reconstitution. Moreover, the observation of Johnson and Kirkwood [3], that membrane extrinsic proteins apparently control membrane resealing supports the hypothesis that the ionizable groups involved are polar groups of proteins. Our results on chymotrypsin-digested ghosts provide addtional support for this hypothesis. In fact, the removal of extrinsic membrane proteins produces the formation of sealed vesicles (Fig. 5). Similarly, treatment of unsealed ghosts with alkaline pH, which reportedly removes spectrin–actin network [32], produces the formation of inside-out microvesicles [52] that appeared sealed (impermeable to FD, data not shown). Both these results indicate that the bilayer interruption, produced at low temperature and ionic strength, is dependent upon the presence of cytoskeletal proteins. On the other hand, the observation that chymotrypsin-digested ghosts can be lysed below pH 5 suggests that charged phospholipids (mainly phosphatidylserine) could also be involved in the resealing in addition to the polar groups of proteins.

In an attempt to correlate the role of temperature and proteins in the resealing process, membrane model systems have been studied. In particular, we studied ionotropic properties of liposomes than can modify the permeability of FD. Briefly, the data are compatible with a lateral phase separation of charged phospholipids from neutral lipids, an event that may account for the phenomena observed in RBC ghosts. Model membranes composed of 1:1 mixture of charged (phosphatidylserine) and uncharged (phosphatidylcholine) phospholipids are permeable to FD if phosphatidylserine lateral phase separation is induced by acidic pH or polycationic proteins. In this respect it is interesting to note that a similar pH effect on membrane permeability to FD has been observed in chymotrypsin-digested ghosts (Figs. 6,7).

To account for the role of temperature in the resealing process, we have tested whether the bilayer interruption and reconstitution could be regulated by a gel–liquid phase transition of particular membrane domains. An evident break in the freedom of motion (related to membrane fluidity) of the 16 NS spin probe has been detected in RBC membrane (Fig. 9). This fluidity break is not detected in chymotrypsin-digested ghosts, although these membranes show, above 15°C, a fluidity similar to that of untreated ghosts. In this respect it is noteworthy that by using the same spin label, RBC of patients with Duchenne muscular dystrophy showed, between 15°C and 40°C, a membrane fluidity similar to that of normal subjects. Yet they did not show any fluidity break around 10°C [8,48]. This was attributed to a different structural organization of pathological membranes.

These results provide evidence that the fluidity break is not an artifact due to the slow motion of the label in RBC membranes (see ref. [53] for a complete discussion), but is indicative of a gel–liquid phase transition. In addition, the presence of a gel–liquid phase transition in RBC membrane is in accordance with data obtained by other EPR studies [5,8,48] or by different physicochemical techniques [4,9,10,54,55]. A 20°C phase transition studied by ^{31}P-NMR linewidth changes in RBC ghosts has been described [6,7]. At variance with data reported in this paper, the ^{31}P-NMR linewidth change is not observed in intact RBC and is remarkably increased after proteolytic treatments of ghosts [7]. The phase transition monitored by ^{31}P-NMR spectra could be due to lipid domains in which apolar protein–lipid interactions are strengthened after digestion of proteins experiencing polar interactions with the bilayer. Moreover, ^{2}H-NMR techniques reported by Davis et al. [56] were unable to detect any gel–liquid phase transition in RBC membrane in the 10°–20°C range. One possible explanation of these inconsistencies has been offered by Verma and Wallach [54] who said that the diverse experimental approaches used may monitor different properties of a composite membrane.

The temperature at which the fluidity break is observed and that at which the resealing begins are very close to each other. Moreover, chymotrypsin treatment of ghosts produces both membrane resealing and the disappearance of the fluidity break. These findings suggest that phase-transition and membrane resealing may be correlated.

Taking together data reported on (a) RBC membrane resealing, (b) model membranes, and (c) fluidity studies, we propose a phase transition model of resealing. The resealing seems to be regulated by a protein-dependent lipid lateral phase separation, induced by low temperature, and increased by low ionic strength. This produces the formation of stable holes through which macromolecules can freely cross. The disorganization of the phase separation produces lipid mixing and membrane reconstitution.

Resealing can be facilitated or induced by: (i) temperature, which induces gel–liquid phase transition and favors lipid–lipid mixing; (ii) salts, that decrease polar protein–lipid interaction; (iii) the removal of proteins that experience polar interactions with charged phospholipids by alkaline or protease treatments. The role of proteins is to maintain the lateral phase separation at low temperature; however, in order to obtain membrane lysis it is also necessary to lower simultaneously the ionic strength. Under these conditions protein–lipid polar interactions could be strengthened and the holes formed during the hemolysis stabilized. We observed, in fact, that in both intact RBC and isotonically resealed ghosts the spin label indicates

the formation of gel-liquid domains at low temperature: lysis, however, was obtained only after lowering of ionic strength. Moreover, this observation suggests that RBC lysis is a reversible phenomenon only if the formation of gel domains is induced at low temperature; where this is true, the hemolysis could be seen also as a phase transition process as hypothesized by Makowki [57].

Looking at the gel-liquid phase transition, it may be suggested that protein-lipid polar domains have characteristic thermotropic properties with a phase transition around 10–15°C. After protein removal the same lipid domains at 0°C are in the liquid state and consequently, no phase transition at 11°C can be detected in liposomes derived from RBC lipids [7,37–39] or in chymotrypsin-digested ghosts (Fig. 9). Protein-lipid polar interactions can modify, in fact, thermotropic properties of lipids [44], for example poly-L-lysine can increase temperature of the gel-liquid phase transition of charged phospholipids [46].

The data reported in this paper indicate that membrane components involved in the resealing process are located at the inner layer of membrane. Phosphatidylserine and cytoskeletal proteins, seem to play the major role. In fact, it is generally accepted that (i) phosphatidylserine is almost completely located at the inner surface of RBC [58,59], (ii) protein removal by alkaline pH produces the availability of phosphatidylserine to cross-linking agents [60], (iii) the association of spectrin-actin network with RBC membrane seems to occur through polar interactions with anchoring proteins [61] and charged lipids. The interaction with polar lipids is supported, besides the mentioned cross-linking studies [60], by evidence obtained with model membranes [62], and by the effects of oxidizing agents on spectrin resulting in changes of phospholipid orientation [63].

Even if charged lipids and cytoskeletal components may be crucial factors in membrane permeability barrier reconstitution, a satisfactory resealing model cannot neglect other contributions. For example, phosphatidylethanolamine that seems to interact with membrane proteins [60] as well as the effects of temperature on conformation and membrane association of cytoskeletal proteins must be mentioned [64,65]. All these factors could be important in protein-lipid association and in phospholipid asymmetry preservation [63].

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