STABILIZATION MECHANISMS OF TRANSBILAYER PHOSPHOLIPID ASYMMETRY IN ERYTHROCYTE MEMBRANE

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ABSTRACT

Studies carried out during the last several years in the author's laboratory on the mechanism(s) of maintenance of the erythrocyte membrane phospholipid asymmetry have been reviewed. The results indicate that this asymmetry in the cells is stabilized mainly by the interactions of membrane skeleton with the overlying membrane bilayer, with or without the involvement of an ATP-driven out-to-in aminophospholipid pump.

INTRODUCTION

One of the most remarkable structural features of biological membranes is that their chemical constituents are asymmetrically distributed in the two halves of their membrane bilayer[1,2]. While this asymmetry is absolute for membrane-bound proteins and carbohydrates, virtually every type of phospholipid is present in both the monolayers, albeit in unequal amounts (figure 1). For example, in mammalian erythrocytes, choline-containing phospholipids [phosphatidylcholine (PC) and sphingomyelin (SM)] are located mainly in the outer half of the membrane bilayer, whereas aminophospholipids [phosphatidylethanolamine (PE) and phosphatidylserine (PS)] are present almost exclusively in the inner monolayer[3]. This phospholipid asymmetry in these cells seems essential not only for maintaining the normal membrane structure and function, but also to circumvent both the red cell destruction by the spleen[4] and hyperactivation of the blood coagulation system[5]. It is, however, not yet clear how an erythrocyte, despite phospholipid diffusion across the membrane[6], maintains the asymmetric membrane phospholipid distribution throughout its life span in the circulation.

To understand the factors that determine the transbilayer phospholipid asymmetry in the erythrocyte membrane, we have studied the membrane phospholipid organization in pathologic/modified erythrocytes[7–16] as well as in unilamellar vesicles formed from natural and/or synthetic phospholipids[17–19]. Here, we present a brief review of these studies along with a possible mechanism underlying the generation and maintenance of the membrane phospholipid asymmetry in mammalian erythrocytes. Prior to discussing our work, we give below a brief account of the red cell membrane structure to orientate the readers who are not familiar with the subject.

The red cell membrane is composed of two major structural units, the membrane bilayer and the underlying meshwork of peripheral membrane proteins, called membrane-associated cytoskeleton or membrane skeleton. Alone, the bilayer has little structural strength and fragments readily by vesiculation. However, its association with the membrane skeleton provides stabilization and resistance to deformation.

The membrane bilayer is analogous to the fluid mosaic membrane of Singer and Nicolson[20], and is formed from the lipid bilayer and integral membrane proteins. The lipid bilayer contains phospholipids and cholesterol as the major lipid constituents, which are present in almost equimolar amounts in human erythrocyte membrane. There are four major phospholipid components (viz. PC, SM, PE and PS) in this membrane[21]; the amounts of choline-containing phospholipids are greater than the aminophospholipids. All these phospholipids are asymmetrically distributed in the two leaflets of the lipid bilayer. While the outer

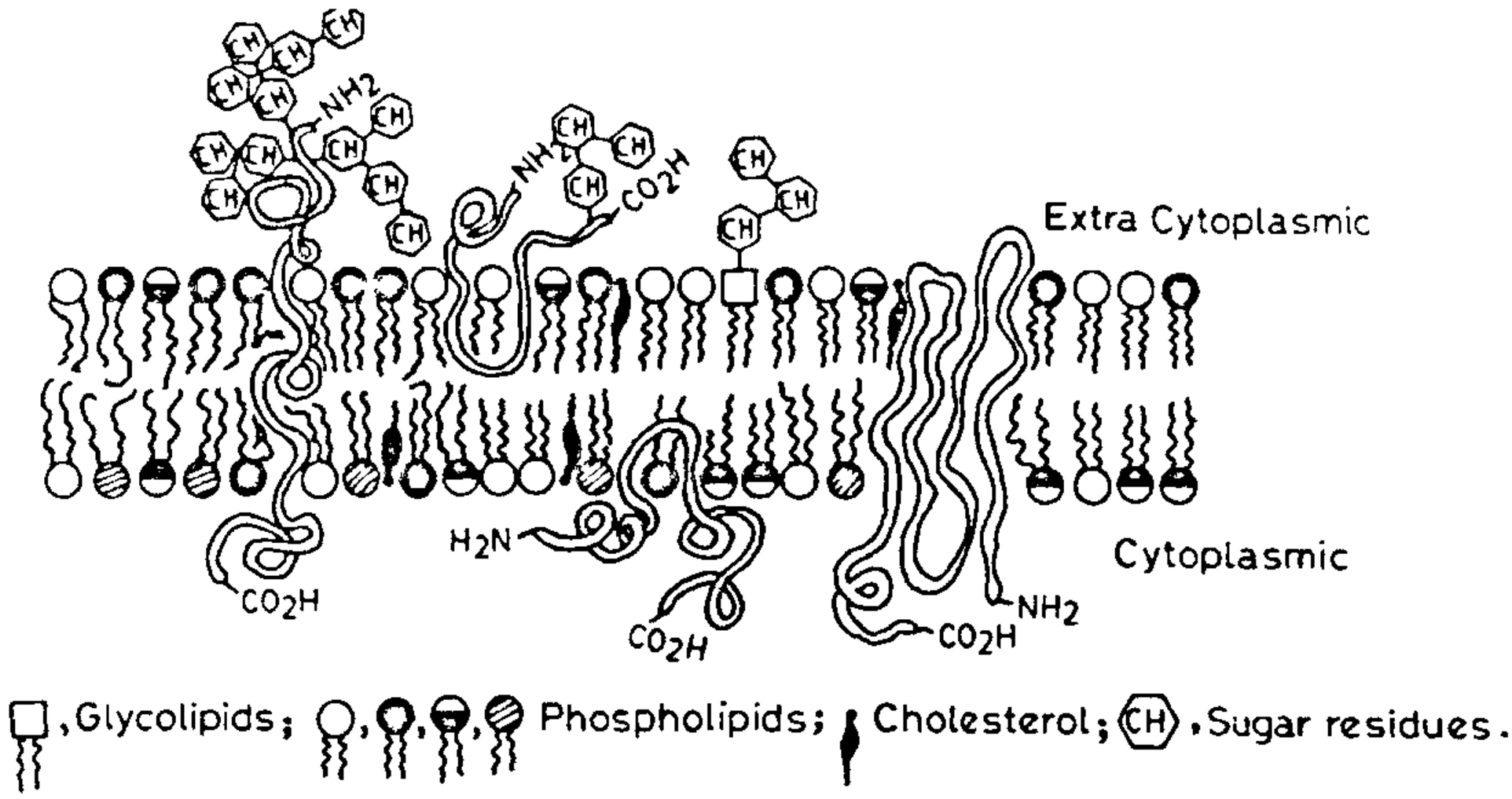

Figure 1. Schematic diagram illustrating asymmetric transbilayer distribution of various membrane components in plasma membranes. Note that total carbohydrate residues bound to proteins and lipids are located exclusively on the outer surface. Also, all the copies of a given membrane protein have only one given orientation. However, most of the phospholipids are found in both the surfaces, albeit in unequal amounts.

monolayer contains about 75% PC, 80% SM, 20% PE and 0% PS, approximately 25% PC, 20% SM, 80% PE and 100% PS are localized in the inner monolayer[2]. It has been suggested that this asymmetric distribution of phospholipids is primarily responsible for the differences in the fluidities of the two surfaces of the erythrocyte membrane bilayer[22], since the fatty acyl chains in PE and PS are more unsaturated than in PC and SM[23].

Anion channel protein (band 3, figure 2B) and glycophorin are the two major integral membrane proteins in the erythrocyte membrane. The band 3 protein has a molecular weight of about 95 KDa and is a mixture of several glycoproteins that are involved in anion transport[24]. The heterogeneity is in the carbohydrate region rather than the polypeptide part[24]. The protein comprises of an intracellular N-terminal domain of about 40 KDa, to which the erythrocyte membrane skeleton, many glycolytic enzymes and haemoglobin are known to bind[25]. The anion transport activity is associated with the C-terminal part of the molecule[26] to which all the known inhibitors of anion exchange are known to bind. The oligosaccharide chain is attached to the protein extracellularly close to its C-terminus[27].

Glycophorins are a class of sialic acid-rich integral membrane proteins, which can be visualized on sodium dodecyl sulphate-polyacrylamide gel electrophoretograms of erythrocyte membrane proteins after periodic acid Schiff's staining. There are at least three types of glycophorins[28], viz glycophorin A, B and C (or D). Amongst these, glycophorin A is the major transmembrane sialoglycoprotein present in the erythrocyte membrane, and has a molecular weight of about 30–50 KDa[29]. Glycophorin B and C are the minor components which possess some peptide regions with amino acid sequence identical to glycophorin A[30,31].

The mammalian erythrocyte membrane skeleton is composed of three major [viz bands 1 and 2 (spectrin), band 5 (actin), and band 4.1 polypeptide] and several minor peripheral membrane proteins[32,33]. Among these, spectrin alone accounts for about 75% of the total membrane skeletal protein mass. This protein is known to consist of two non-identical subunits, now generally referred to as α (Mr 240 KDa) and β (Mr 220 KDa), and exists *in vivo* as linear tetramers and branched higher order oligomers[34]. The smaller subunit (band

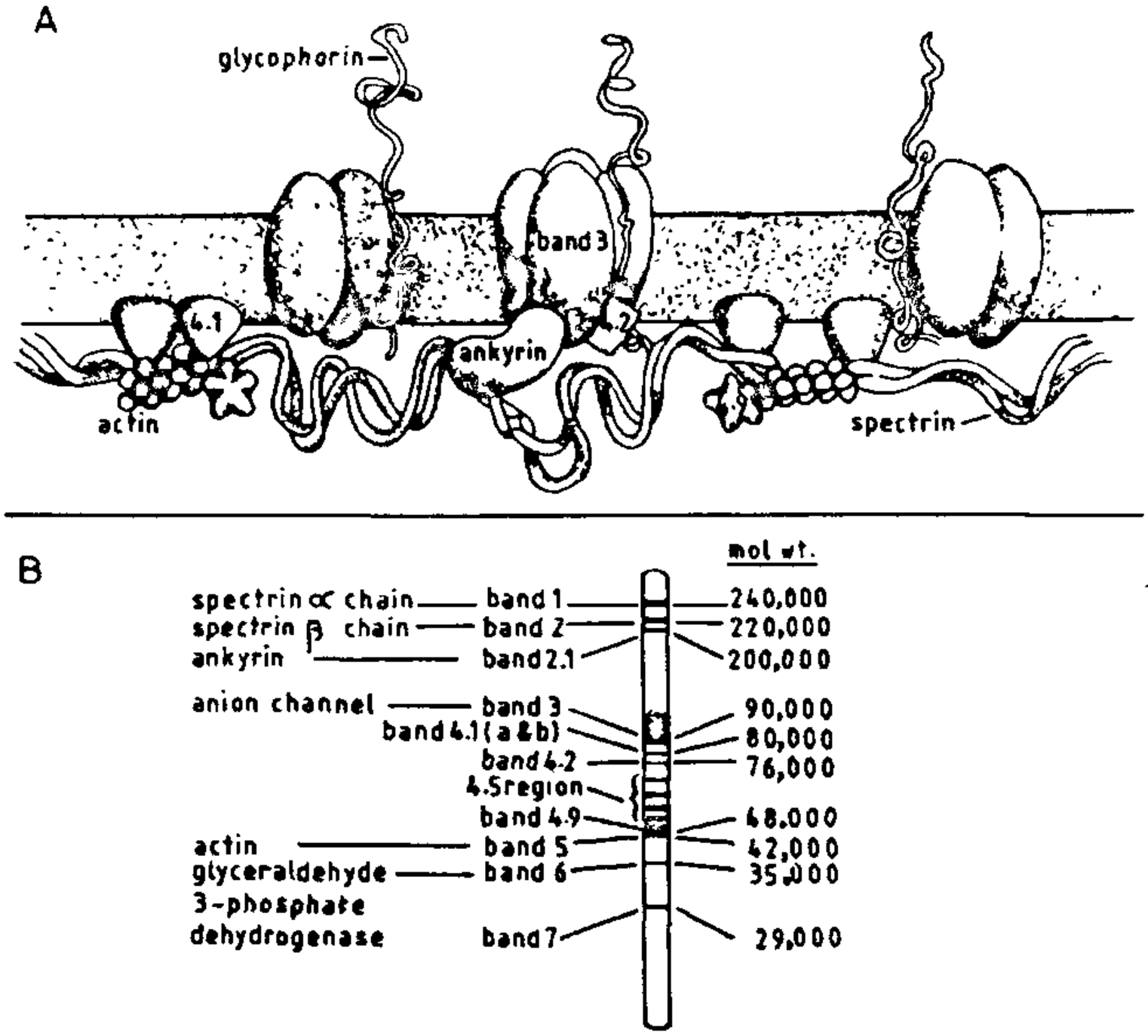

Figure 2A-B. **A.** Schematic diagram illustrating the transmembrane distribution and molecular associations of the major red cell membrane proteins. The extracellular medium comprises the upper half of this figure. **B.** Schematic diagram of a 5% polyacrylamide–sodium dodecyl sulphate gel of red cell membrane proteins run according to the method of Fairbanks *et al*[67] (redrawn from ref. 68).

2) is phosphorylated at four closely spaced sites near its C-terminus[34]. Both the subunits contain unique, multiple, proteolytically-resistant domains connected to each other by small protease sensitive regions[35]. The skeletal network is formed by attachment of spectrin tetramers at their ends to multiple junction points, consisting of short filaments of F-actin, each containing some 15–20 monomers[32]. Participation of the band 4.1 polypeptide in this attachment strengthens the binding of spectrin to F-actin[36]

The membrane skeleton is associated with the overlying membrane bilayer primarily through bands 2.1 and 4.1 proteins. Band 2.1 protein (ankyrin) is comprised of two structural domains, one with high affinity for binding sites on the spectrin molecule[37] and the other contains a binding site for the cytoplasmic part of the anion channel protein[38] (figure 2A). Band 4.1 protein, on one hand, is linked to the spectrin-actin complex[32, 33] and on the other, with cytoplasmic tail of glycophorin[39]. Also, this protein is known to bind to the anion channel protein, though with lower affinity[40].

The membrane skeleton is responsible for many of the structural properties of the erythrocyte membrane. Not only does the skeleton control the membrane deformability, but also has a central role in controlling the glycoprotein lateral distribution and mobility[41]. Most of the perturbations at the cell surface cause alterations in the membrane skeleton properties, either directly through shear distortions or indirectly through alterations in cellular metabolism[41]. From the fact that cell recovers the normal skeletal properties, which may be necessary for viability, it appears that the membrane properties are controlled by the rate of breaking and remaking of the interactions in the membrane skeleton.

EXPERIMENTAL PROCEDURES

A number of methods have been used to ascertain the transbilayer phospholipid distribution in the erythrocyte (or unilamellar vesicles) membrane[42], but in this article we shall discuss only those techniques which we have employed in our laboratory. We have primarily used the following three types of techniques to probe the membrane phospholipid organization: (i) enzymatic techniques, (ii) chemical labelling techniques, and (iii) Merocyanine 540 staining.

Phospholipases have been commonly used as enzymatic probes to analyse the transbilayer phospholipid distribution[43]. In our studies, we have employed phospholipase A_2, which is known selectively to cleave the C–2 ester bond in glycerophospholipids[43]. The lyso-phospholipids and fatty acids so generated in the outer monolayer of the membrane bilayer do not have a lytic effect on erythrocytes and vesicles, and stay within the membrane[44]. Since specificity of this enzyme towards phospholipids depends mainly on the source from which it is isolated, we have used phospholipases A_2 from *Naja naja* snake venom[7–9,18,19], bee venom[10,11,13–16] and porcine pancreas[9–11,13–16] to overcome this problem. *Naja naja* phospholipase A_2 degrades PE faster than PC and PS, whereas pancreatic phospholipase A_2 hydrolyses PS faster than PC and PE. However, both PC and PE are degraded at almost equal rate by phospholipase A_2 from bee venom.

Accessibility of the various membrane glycerophospholipids to phospholipase A_2 in intact cells has generally been correlated with their localization in the outer leaflet of the membrane bilayer[2,42,43]. However, in some cases the phospholipase A_2 treatment has been shown to induce reorganization of membrane phospholipids in modified erythrocytes[45,46]. It is, therefore, advisable to cross-check the data using an alternative technique. Keeping this in view, we employed amino-group labelling reagents[2,42,43] as external membrane probes to analyse the membrane phospholipid distribution. Trinitrobenzenesulphonic acid[7,8,10,12,17,18] and fluorescamine[14,16] were used for labelling the amino-groups of PE and PS in intact cells (or vesicles). Care was taken to stop the penetration of the reagents into the cells/vesicles. Since the reaction time for fluorescamine is much shorter[46] as compared to trinitrobenzenesulphonic acid[2], it is often advisable to use fluorescamine for ascertaining the aminophospholipid distribution across the erythrocyte membrane.

In the above two techniques, the cells were treated with appropriate enzymes/amino group-labelling reagents under carefully-controlled conditions such that the reagents modified only the external membrane phospholipids in the intact erythrocytes/vesicles. Also, in each study separate experiments were carried out to confirm that the reagents did not penetrate into the cells or lysed the vesicles.

Together with the above methods, we have also used the 'fluid-sensing' fluorescent dye, Merocyanine 540, for assessing the transbilayer phospholipid distribution in erythrocytes. This dye in the presence of 2–5% serum does not stain the normal erythrocytes, but it readily labels those red cells which have undergone significant changes in transbilayer organization of their membrane phospholipids[22]. As in-to-out migration of membrane aminophospholipids in erythrocytes should lead to enhanced outer surface fluidity[23], the cells having an altered phospholipid distribution across their membranes would readily be stained by Merocyanine 540.

RESULTS AND DISCUSSION

To investigate the role of lipid-lipid interactions in determining the membrane phospholipid asymmetry, we studied the transbilayer phospholipid distribution in unilamellar vesicles comprised of purified phospholipid components (PC, PE, SM and PS) of the red cell membrane[18]. The vesicles were formed by sonication and detergent dialysis, and had outer diameters of 30 nm and 45 nm respectively. In 30 nm outer diameter vesicles, PS was found to prefer the inner monolayer whereas PC and PE distributed almost symmetrically across the vesicles bilayer. This PS

asymmetry, however, disappeared upon increasing the vesicle outer diameter to 45 nm, suggesting that the PS asymmetry in small vesicles (O.D. 30 nm) was enforced by the high degree of surface curvature rather than the specific inter lipid interactions. To examine whether the inclusion of cholesterol in vesicles bilayer would influence the transbilayer phospholipid distribution, we analysed the phospholipid organization in both the types of vesicles formed from the phospholipids and 40 weight % cholesterol[18]. The phospholipid distribution across the vesicles bilayer remained virtually unaffected by the inclusion of cholesterol in the phospholipid bilayers. These results strongly indicate that phospholipid-phospholipid and phospholipid-cholesterol interactions play no significant role in determining the membrane phospholipid asymmetry in erythrocytes[18].

As an extension of these studies, we also analysed the structural parameters of phospholipids which control their distribution across the small vesicles bilayer. Structurally-modified PE and PC were synthesized, and their transbilayer distributions were studied in small vesicles containing natural PC or SM. Results of these studies have revealed that transbilayer distribution of the various phospholipids in curved vesicles is determined mainly by their effective polar head-group volume rather than the head-group charge or length[17,19].

Our first and most useful approach to analyse the factors that determine the erythrocyte membrane phospholipid asymmetry has been the studies of those pathologic erythrocytes which we suspected to have structural defects in their membrane skeleton. The main consideration in selecting such red cells was based on the studies of Haest *et al*[47], who showed that treatment of human erythrocytes with sulphydryl oxidizing agents leads to structural defects in the major membrane skeletal protein, spectrin, as well as loss of the membrane phospholipid asymmetry. In this context, we undertook studies on membrane phospholipid organization and membrane skeleton in red cells of both malaria-infected animals and humans suffering from chronic myeloid leukaemia. The results of these studies are summarized as below:

Several studies have suggested that blood stage malarial parasite induces marked structural changes in membrane skeleton of the infected erythrocytes (reviewed in refs. 48, 49). Although these changes have not yet been demonstrated at the molecular level, there is every reason to believe (see ref. 48) that the parasite must structurally modify the erythrocyte membrane skeleton to facilitate its entry and subsequent growth inside the host cells. We, therefore, analysed the membrane phospholipid organization in erythrocytes harbouring different developmental stages of the simian malarial parasite *Plasmodium knowlesi* or the human parasite *P. falciparum*.

Significant changes in transbilayer organization of membrane phospholipids have been observed in monkey erythrocytes infected with *P. knowlesi*. These changes become quite apparent even at the parasite developmental stage that follows soon after invasion[7]. At this stage, PE was found to partially migrate from the inner to the outer monolayer, and the phospholipid loss in the inner monolayer was compensated by a reverse movement of PC from the outer monolayer. Further studies have shown that changes in the transbilayer organization of membrane phospholipids increase with the parasite maturation inside the infected erythrocytes[13]. Together with increasing amounts of PE, a considerable fraction of PS also moved from the inner to the outer surface of red cells infected with *P. knowlesi* trophozoites or schizonts. These membrane changes were not limited to *P. knowlesi*-infected monkey red cells, as we have observed similar membrane alterations also in the human erythrocytes harbouring different developmental stages of *P. falciparum*[15].

Besides the parasitized red cells, the membrane phospholipid asymmetry was disturbed also in the nonparasitized erythrocytes of some *P. knowlesi*-infected monkeys[8,11]. This membrane change in these cells was accompanied[8]

by a structural alteration in spectrin and an increased level of intracellular Ca^{2+}.

Structurally abnormal spectrin has also been detected in red cells of humans suffering from chronic myeloid leukaemia[9]. The two subunits of this protein appeared to undergo crosslinking, via disulphide bonds, due to decreased levels of intracellular glutathione[9]. This abnormality in spectrin was found to be accompanied by changes in transbilayer organization of membrane phospholipids in these erythrocytes[9,12].

The above studies have suggested that the membrane phospholipid asymmetry in erythrocytes is determined not by the phospholipid-phospholipid and phospholipid-cholesterol interactions[18], but it seems to originate from preferential interactions of inner layer phospholipids with the underlying membrane skeletal proteins, especially spectrin[9,10]. This was quite consistent with the findings that spectrin binds preferentially to PS in mixed phospholipid bilayers[50-52]. To understand further the role of this protein in maintaining the erythrocyte membrane phospholipid asymmetry, we have structurally modified spectrin in both monkey and human red cells, and then analysed the membrane phospholipid organization and membrane protein structure in the modified cells. Spectrin in intact erythrocytes was structurally-altered[10] by heating the cells at the thermal transition temperature (49–50°C) of the protein. It has been suggested earlier[53,54] that at this temperature only those structural regions of human erythrocyte membrane spectrin which do not directly interact with the membrane are modified, while the regions which interact with the membrane remain essentially unaltered.

Heating of the human red cells at 49–50°C for 15 min did not induce any change in transbilayer organization of their membrane phospholipids[16], but an identical treatment of the monkey red cells resulted in migration of PE and PS from the inner to the outer monolayer[10,16], though in both the cases the cell shape was transformed from discocytes to predominantly spherocytes[16]. These alterations in red cells were accompanied by some distinct changes in structure and composition of membrane proteins. Apart from partial irreversible denaturation of spectrin, tendency of several cytosolic proteins to tightly associate with the membrane was markedly enhanced by heating both the human and monkey erythrocytes. Although the extent of spectrin denaturation in monkey cells was similar to that observed in human erythrocytes, at least two clear differences were seen between the membrane protein patterns of heated human and monkey cells (C. M. Gupta, S. M. Gokhale, A. Kumar and V. Bhakuni, unpublished work). First, unlike heated human erythrocytes, an approximately 70 KDa protein, which was a major cytosolic protein in normal monkey red cells but not in the normal human erythrocytes, was one among the four major cytosolic proteins that were tightly associated with the heated monkey red cell membrane; the remaining three major proteins were similar to those present in the membranes isolated from heated human erythrocytes. Second, the intensity of polypeptide 4.1 and a 150 KDa membrane protein appeared to be reduced in the membranes of heated monkey cells but not in the heated human erythrocyte membrane. Furthermore, the 70 KDa cytosolic protein in the heated monkey erythrocytes was found to associate with the membrane skeleton rather than the membrane bilayer. These results have been interpreted to suggest that: (i) at least the structural regions of spectrin which are not directly attached to the overlying bilayer play probably no role in maintaining the phospholipid asymmetry, and (ii) together with spectrin, the membrane skeleton dynamics (or structural integrity) and the membrane proteins that anchor the membrane skeleton to the bilayer (e.g. polypeptide 4.1 and ankyrins) play an important role in stabilizing the preferential distribution of aminophospholipids in the inner monolayer.

Defects in the membrane skeleton dynamics and structure of anchoring proteins should not only affect the structural integrity of the membrane skeleton but also the skeleton-

membrane bilayer associations[33,34,39,40], which in turn would result in weakening[55] of the aminophospholipid interactions with polypeptide 4.1[56] and spectrin[50–52]. It may, therefore, be considered that the observed loss of phospholipid asymmetry in the heated monkey erythrocytes is induced probably by the dissociation of the spectrin-actin complex from the overlying membrane bilayer. This dissociation, on one hand, would make the aminophospholipids free to diffuse across the membrane bilayer while on the other, it could lead to reorganization of integral membrane proteins[41]. As an altered integral membrane protein distribution may induce structural deformities which could serve as leakage sites for phospholipids within the membrane[57,58], it is suggested that in-to-out migration of aminophospholipids in the erythrocyte membrane is controlled mainly by the membrane bilayer-skeleton associations.

To examine further the role of anchoring proteins in maintaining the erythrocyte membrane phospholipid asymmetry, we have studied the membrane phospholipid organization in Ca^{2+}—loaded human erythrocytes[14]. Although several erythrocyte membrane proteins have been shown to be sensitive to the intracellular-free Ca^{2+} levels[59–62], ankyrin (band 2.1) seems to be the most susceptible protein to proteolytic degradation by Ca^{2+} activated proteases[59,61]. Since degradation of ankyrin would lead to dissociation of the membrane skeleton from the membrane bilayer (see figure 2A), the red cells containing degraded ankyrin are expected to have abnormal phospholipid distribution across their membranes. To test this possibility, the free Ca^{2+} concentration was so manipulated that it induced almost selective proteolysis of ankyrin in the intact human erythrocytes[14]. As expected, these cells were indeed found to have an altered phospholipid distribution across their membrane bilayer[14].

CONCLUSION

These studies strongly indicate that preferential distribution of aminophospholipids (PE and PS) in the inner monolayer is stabilized by their interactions with skeletal proteins, e.g. spectrin and polypeptide 4.1. Also, it suggests that only those structural regions of spectrin which are directly attached via anchoring proteins (e.g. polypeptides 2.1 and 4.1) to the membrane bilayer may interact with the inner layer phospholipids. These interactions seem to be weak in nature, and may be abolished by inducing alterations in membrane skeleton dynamics and/or in structure of anchoring proteins. An altered anchoring protein structure and/or skeleton dynamics would adversely affect the associations of membrane skeleton with membrane bilayer, which in turn could lead to the release of constraints which these associations impose on the in-to-out aminophospholipid migration, and also to generation of new reorientation sites for phospholipids within the membrane. It is thus quite evident that the membrane skeleton-bilayer interaction is the major factor that restricts the outward diffusion of aminophospholipids within the erythrocyte membrane.

Recent studies of Devaux and coworkers[63–65] have shown that erythrocyte membrane contains an ATP-driven system which transports PE and PS from the outer to the inner monolayer. The transporter seems to translocate PS faster than PE[64,65] and could be inactivated by even 2-fold increase in intracellular-free Ca^{2+} concentration[65] or by treating the erythrocytes with maleimide[65]. Further, it has been suggested that this transport is responsible for promoting and maintaining the erythrocyte membrane phospholipid asymmetry[63]. Since we observed no change in transbilayer distribution of membrane phospholipids in erythrocytes that received pretreatment with 10 mM maleimide (P. Dubey and C. M. Gupta, unpublished work) or had 2–3-fold greater intracellular-free Ca^{2+} concentration (R. Chandra and C. M. Gupta, unpublished work), it is inferred that ATP-driven out-to-in aminophospholipid transport alone is not sufficient for maintaining the membrane phospholipid asymmetry. This is well-supported by the recent suggestion[66] that

the erythrocyte membrane lipid pump, first reported by Seigneuret and Devaux[63], represents mainly the activity of an ATP-dependent lipid flip/flop catalyst rather than a mechanism for maintenance of asymmetric phospholipid distribution across the erythrocyte membrane. We, therefore, conclude that differential binding of phospholipids with membrane skeletal proteins, alone or together with the lipid pump[63], is the major determinant of transbilayer phospholipid asymmetry in red cells.

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NEWS

STUDY OF SOLAR FLARES

The visibility of solar flares from the Earth is the ultimate stage of an explosion taking place on the Sun, and not the beginning as was thought until recently, reports Anna Frank, associate of the USSR Academy Institute of General Physics.

He says that the explosion is caused by an over concentration of magnetic energy in one point, a resultant acceleration of charged particles and their ejection into outer space.

Solar flares have been the subject of increasing scientific interest. They tell negatively on people's disposition, interrupt radio communication and their forecast is important for space-flights' safety. Study of solar flares is expected to provide the key to many mysterious phenomena in the Universe (*Soviet Features*: Science and Technology, Vol. XXVI. No. 123; Published by: Information Department, USSR Embassy in India, P.B. 241, 25 Barakhamba Road, New Delhi 110 001.)