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# Role of membrane-associated cytoskeleton in maintenance of membrane structure*

C. M. GUPTA†, A. KUMAR and P. JOSHI
Division of Biophysics, Central Drug Research Institute, Lucknow 226 001, India

**Abstract.** Various structural components of biological membranes are asymmetrically localized in the two surfaces of the membrane bilayer. This asymmetry is absolute for membrane (glyco) proteins, but only a partial asymmetry has been observed for membrane phospholipids. In the red cell membrane, choline-phospholipids are localized mainly in the outer monolayer whereas aminophospholipids are distributed almost exclusively in the inner monolayer. Several evidences are now available to suggest that this distribution of membrane phospholipids in red cells is directly or indirectly maintained by the membrane-associated cytoskeleton (membrane skeleton). This belief is well supported by the previous as well as recent studies carried out in the authors laboratory. Previously, it has been shown that lipid-lipid interactions play no major role in maintaining the transmembrane phospholipid asymmetry in erythrocytes, and that the asymmetry is lost upon covalent crosslinking of the major membrane skeletal protein, spectrin. The recent data presented here further shows that degradation or denaturation of spectrin indices rapid transbilayer movement of membrane phospholipids in the cells which, in turn, leads to more random phospholipid distributions across the membrane. These studies taken together strongly suggest that the skeleton-membrane associations are the major determinants of the transmembrane phospholipid asymmetry in erythrocytes, and that the dissociation of the skeleton from the membrane bilayer probably results in generation of new reorientation sites for phospholipids in the membrane.

**Keywords.** Cytoskeleton; membrane bilayer; phospholipid asymmetry; phospholipases; erythrocytes.

## Introduction

It is now commonly believed that the cell cytoskeleton, by virtue of its interactions with the plasma membrane, not only regulates the membrane function but also controls the membrane structure. Several dynamic processes like cell motility, cell-cell interactions, membrane deformability, endocytosis, lateral diffusibility of membrane integral proteins, etc., seem to be governed by the cytoskeleton-membrane interactions (reviewed by Geiger, 1983). Amongst the various types of cells studied so far, the red cell cytoskeleton and its membrane associations is the best characterized system (for reviews see: Haest, 1982; Cohen, 1983; Marchesi, 1983; Sheetz, 1983).

The red cell membrane is composed of two structural units, *viz*. the membrane

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† To whom correspondence should be addressed.

Abbreviations used: CML, Chronic myeloid leukaemia; SDS, sodium dodecyl sulphate; CD, circular dichroism; TNBS, trinitrobenzenesulfonic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; DTNB, 5,5′-dithio bis(2-nitrobenzoic acid); PC, phosphatidylcholine.

bilayer and the reticulate structure of membrane peripheral proteins, called membrane skeleton. The membrane bilayer alone has very little mechanical strength and fragments readily by vesiculation. The membrane skeleton associates with the cytoplasmic face of the bilayer, and this association provides mechanical stability to the membrane and also regulates the membrane structure and function (Sheetz, 1983). The skeleton is composed of three major (spectrin, actin and polypeptide 4·1) and possibly several minor proteins which specifically interact to form a reticulate type of structure (Cohen, 1983). Of the three major proteins, spectrin (Bands 1 and 2) alone accounts for about 75% of the total skeletal protein mass (Marchesi, 1983).

The skeletal network associates with the membrane bilayer primarily *via* protein–protein interactions (Cohen, 1983; Anderson and Lovrein, 1984). This association seems to be further stabilized by the interactions of the inner layer phospholipids with the underlying skeleton (Haest, 1982). The skeleton-membrane interactions not only control the lateral diffusion of membrane integral proteins (Sheetz, 1983), but also appear to maintain the asymmetric transmembrane organization of phospholipids in the erythrocytes (Haest *et al*., 1978; Lubin *et al*., 1981; Gupta *et al*., 1982; Kumar and Gupta, 1983; Schwartz *et al*., 1984; Kumar and Gupta, 1984). The work presented here clearly shows that on dissociating the skeleton from the membrane bilayer, by inducing structural changes in the skeletal components, results in the rapid transbilayer movements of phospholipids in the intact cells. This in turn leads to more random phospholipid distribution across the membrane.

## Experimental procedures

Role of the membrane skeleton in the maintenance of the transmembrane phospholipid organization was ascertained by analysing the transbilayer phospholipid distributions in the red cells that possessed abnormalities in their skeleton. These cells include both pathologic erythrocytes and the red cells in which defined skeletal defects were induced *in vitro*. The pathologic erythrocytes studied were the red cells of humans suffering from chronic myeloid leukaemia (CML erythrocytes) as well as the rhesus monkey red cells harbouring schizont stage of the malarial parasite, *P. knowlesi.* Detailed procedures for isolation and purification of these cells and their membranes have already been published elsewhere (Gupta and Mishra, 1981; Kumar and Gupta, 1983). Membranes of these cells were routinely analysed for their protein compositions by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (Fairbanks *et al*., 1971).

Skeletal defects in the monkey red cells were induced *in vitro* by subjecting these cells to brief heat treatments at the helix-coil transition temperature of spectrin (Brandts *et al*., 1977). The heating was carried out essentially according to Palek *et al*. (1981). The extent of spectrin denaturation was determined by circular dichroism (CD). Spectrin samples for the CD measurements were prepared as described by Smith and Palek (1983).

The methods that are currently in use for studying the transmembrane phospholipid organization can be classified into four general categories: (i) enzymatic hydrolysis, (ii) chemical labelling, (iii) immunochemical labelling, and (iv) biophysical techniques (reviewed by Etemadi, 1980). Amongst these, enzymatic (*e.g*., phospholipase $A_2$ and

sphingomyelinase) and chemical (*e.g.*, trinitrobenzene sulphonic acid (TNBS) and fluorescamine) probes have been most commonly used (reviewed by Roelofsen, 1982). The essential requirements during the use of these probes in such studies are that the chemical reagents/enzymes must not permeate across the membrane, and that they should completely label/hydrolyse the external phospholipids without affecting the cell integrity. Keeping in view these requirements, we have optimised the conditions for using phospholipase $A_2$, from various sources, and TNBS to probe the membrane phospholipid organization. Experimental procedures in detail have been described previously (Gupta and Mishra, 1981; Gupta *et al.*, 1982; Kumar and Gupta, 1983,1984).

## Results

### *Membrane phospholipid organization in red cells having cross-linked spectrin*

In our earlier studies (Kumar and Gupta, 1983), we have shown that CML erythrocyte spectrin is crosslinked *via* disulphide bonds. This abnormality in these cells was associated with loss of the transmembrane phospholipid asymmetry, as analysed by phospholipase $A_2$. The latter observation has now been further confirmed by employing the chemical probe, TNBS. Table 1 shows that unlike the normal human red cells, TNBS labelled both phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the CML erythrocytes. To ensure that the labelling of PS in the CML cells did not result due to permeation of the probe across the membrane (Haest *et al.*, 1981), the red cell anion channel was blocked by the anion channel protein inhibitor, 5,5′-dithio bis(2-nitrobenzoic acid) (DTNB) (Reithmeier, 1983).

**Table 1.** Human red cell aminophospholipid labelling with TNBS at 6 h (20°C).

| Sample | PE (%) | PS (%) |
|---|---|---|
| Normal erythrocytes | 24·1 ± 3·2 | 0 |
| | 19·3 ± 1.1[a] | 0 |
| CML erythrocytes | 39·8 ± 2·5 | 40·8 ± 1·9 |
| | 26·3 ± 0·5[a] | 29·1 ± 0·2 |

[a] Labelling was carried out in the presence of the red cell anion channel protein inhibitor, DTNB (Reithmeier, 1983).Values are mean of 4–6 determinations ± S.D.

### *Membrane phospholipid organization in red cells having degraded spectrin*

Intracellular malarial parasite is well known to cause extensive degradation of the red cell spectrin during the schizont stage of its development (Weidekamm *et al.*, 1973; Wallach and Conley, 1977; Gruenberg and Sherman, 1983). Therefore, we decided to examine the effect of spectrin degradation on the transbilayer distributions of

phosphatidylcholine (PC), PE and PS in the membranes of rhesus monkey red cells that were infected with the mature schizont stage of the simian malarial parasite, *P. knowlesi*, using pancreatic phospholipase $A_2$ as the external membrane probe. Table 2 shows that unlike the normal uninfected monkey red cells, this enzyme readily hydrolysed about 40% PE and 47% PS in the infected cells. This indicates that degradation of spectrin in the intact red cells induces marked reorganization of membrane phospholipids in the two surfaces of the membrane bilayer.

**Table 2.** Red cell phospholipid degradation by phospholipase $A_2$[a] at 1 h.

| Red cells | PC (%) | PE (%) | PS (%) |
|---|---|---|---|
| Normal uninfected monkey red cells | 10·0 ± 1·8 | 0 | 0 |
| Red cells infected with *P. knowlesi* | 25·4 ± 3·9 | 39·4 ± 2·8 | 47·1 ± 2·7 |

[a] From porcine pancreas. Values are mean of 12–16 determinations ± S.D.

*Membrane phospholipid organization in red cells having denatured spectrin*

Previous studies have demonstrated that heating of the human red cells at 48–51°C results in thermal denaturation of spectrin (Brandts *et al*., 1977) and fragmentation of the cells into spherocytes and exocytotic vesicles (Ham *et al*., 1948). Therefore, we heated the monkey red cells at 47°C, 49°C and 51°C. The effect of the heat treatment on the red cell shape was examined by light microscopy. This treatment at 49°C for 15 min led to the formation of micro vesicles and consequently the transformation of the cell shape from diskocytes to spherocytes (data not shown). No such effect on the integrity of the membrane was observed upon heating the cells under identical conditions at 47°C. On the contrary, extensive hemolysis of the cells occurred when the heating temperature was raised to 51°C. To confirm that heating of the monkey red cells at 49–50°C results in irreversible thermal denaturation of spectrin, we determined the apparent helical content of spectrin, both before and after heating, using C.D. Table 3

**Table 3.** Denaturation of spectrin on heating at 49–50°C.

| Heating time (min) | Loss of helical content[a] (%) |
|---|---|
| 5 | 5–8 |
| 10 | 20–25 |
| 15 | 28–30 |

[a] Determined by C.D.

shows that 28–30% of the total helical content of monkey red cell spectrin was lost upon heating at 49–50°C for 15 min. However, this treatment in identical conditions for 10 min and 5 min appeared to result in only 20–25% and 5–8% loss of the apparent helicity respectively. It may, therefore, be concluded that the heating of red cells at 49–50°C leads to unfolding of spectrin.

Effect of spectrin denaturation on the transmembrane phospholipid organization in the red cells was analysed using phospholipase $A_2$, from two different sources, as the external membrane probe. Table 4 shows that both bee venom phospholipase $A_2$ and pancreatic phospholipase $A_2$ hydrolysed greater amounts of membrane phospholipids in the heat-treated cells than in normal cells. In the normal cells, only PC and PE were accessible to bee venom phospholipase $A_2$. But this enzyme in the heated cells also degraded PS. That a fraction of PS from the inner monolayer has moved to the outer monolayer in the heated cells was further confirmed by employing pancreatic phospholipase $A_2$ as the enzymatic probe (Roelofsen, 1982). Unlike the normal red cells, this enzyme readily hydrolysed PC, PE and PS in the heat-treated cells (table 4). These results demonstrate that denaturation of spectrin induces loss of membrane phospholipid asymmetry in erythrocytes.

**Table 4.** Monkey red cell phospholipid hydrolysis by phospholipase $A_2$ at 1 h.

| Sample | Enzyme source | PC (%) | PE (%) | PS (%) |
|---|---|---|---|---|
| Normal red cells | Bee venom | 45·5 ± 0·8 | 21·3 ± 1·2 | 0 |
| | Porcine pancreas | 7·1 ± 1·2 | 0 | 0 |
| 15 min Heat-treated red cells | Bee venom | 41·4 ± 4·6 | 36·1 ± 0·6 | 33·8 ± 2·5 |
| | Porcine pancreas | 39·9 ± 0·1 | 33·6 ± 1·7 | 34·8 ± 0·2 |

Values are mean of 4–6 determinations ± S.D.

## Discussion

In the red cell membrane, the choline-phospholipids localize mainly in the outer monolayer whereas the aminophospholipids distribute almost exclusively in the inner monolayer (Op den Kamp, 1979). It is, however, not yet clear as to how this asymmetric distribution of the membrane phospholipids is generated and maintained in the cells. Our previous studies have shown that lipid–lipid interactions play no major role in the maintenance of the membrane phospholipid asymmetry, and suggested that it probably originates from differential interactions between phospholipids and membrane proteins, especially skeletal proteins (Kumar and Gupta, 1984). This suggestion is well supported by the finding that covalent crosslinking of the red cell spectrin is invariably associated with loss of the transmembrane phospholipid asymmetry (Haest *et al.*, 1978; Gupta *et al.*, 1982; Kumar and Gupta, 1983). Also, random distributions of PS have been observed in the red cells that lack polypeptide 4·1, another major skeletal protein (Schwartz *et al.*, 1984). Moreover, absence of the membrane phospholipid asymmetry has been witnessed in the red cells which have deformed membrane skeleton (Lubin

*et al*., 1981). Furthermore, our present studies clearly demonstrate that covalent crosslinking or degradation or denaturation of spectrin in intact red cells results in more random distributions of membrane phospholipids in the two surfaces of the membrane bilayer. These observations put together provide sufficient evidence to the belief that the membrane skeleton directly or indirectly controls the asymmetric organization of membrane phospholipids in the red cells.

Degradation, denaturation or genetic deficiency of one or more skeletal proteins in red cells must result in either disruption or destabilization of the skeletal network. On the other hand, covalent crosslinking of skeletal proteins would tend to rigidify the skeleton, which in turn will affect the skeletal dynamics (Sheetz, 1983). In either of these cases, the skeleton-membrane associations should be modified. That these associations stabilize the red cell membrane phospholipid asymmetry is further supported by the finding that the transmembrane phospholipid distributions in red cells are altered during drug-induced endocytosis (Schrier *et al*., 1983).

Several studies have shown that skeletal defects in red cells induce rapid transbilayer movements of phospholipids in the membrane (Mohandas *et al*., 1982; Franck *et al*., 1982, 1983; Bergmann *et al*., 1984a). This is quite consistent with our observations that various structural alterations in spectrin lead to migration of PS to the outer surface. To examine whether the rate of these movements is related to the extent of structural changes introduced in the skeleton, we have studied the kinetics of membrane phospholipid hydrolysis in the red cells that received heat treatments for varying periods of time. Figure 1 shows that the amounts of hydrolysed phospholipids in the 15 min and 10 min heat-treated cells (B and C) did not significantly increase with time. But in the 5 min heated cells the hydrolysis of PE and PS increased with time upto 4 h after which it virtually remained constant at least upto 12 h (D, figure 1). These observations clearly show that extensive structural changes in spectrin lead to almost spontaneous transbilayer movements of PE and PS from the inner monolayer to the outer monolayer, however, these movements are relatively slow in case a very limited fraction of the cell spectrin is altered.

Transbilayer phospholipid movement in the normal red cells is a very slow process (van Meer and Op den Kamp, 1982). Acceleration of this process in the cells having altered membrane skeleton should, therefore, arise from some specific membrane changes which facilitate the transbilayer mobility of phospholipids in the membrane. We propose that these membrane changes result in generation of new reorientation sites for phospholipids in the cells. The number of these sites seems to be governed by the extent of the dissociation between skeleton and the bilayer, as suggested by our finding that transmembrane phospholipid mobility in the 15 min heat-treated red cells was considerably faster than in the 5 min heated cells. This contention is further supported by the studies of Dressier *et al.* (1984) wherein it has been shown that the extent of spectrin crosslinking in the diamide-treated red cells is in direct correlation to the rate of transbilayer movements of exogenous phospholipids.

From these discussions, it would appear that the membrane phospholipid asymmetry is stabilized by the skeletal control of reorientation sites for phospholipids in the membrane. But recent studies of Bergmann *et al.* (1984b) have demonstrated that exogenous lyso PS inserted in the outer monolayer of the red cells almost spontaneously concentrates in the inner monolayer, whereas lyso PC prefers the outer

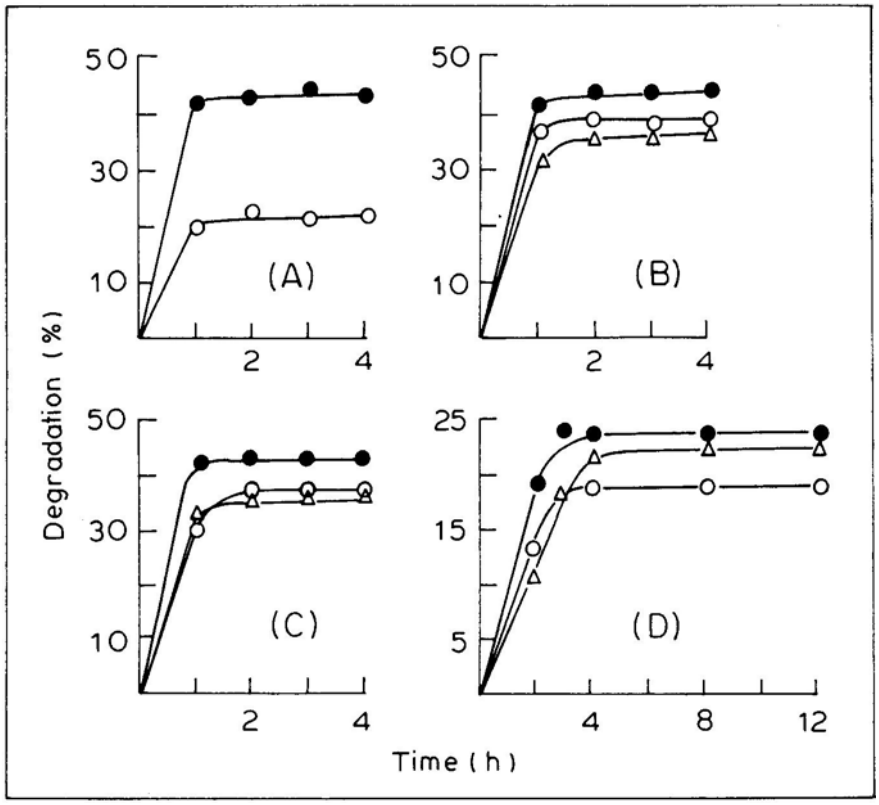


**Figure 1.** Kinetics of hydrolysis of erythrocyte phospholipids by phospholipase $A_2$. A, normal erythrocytes; B, 15 min heat-treated erythrocytes; C, 10 min heat-treated erythrocytes; D, 5 min heat-treated erythrocytes. Heat treatment was carried out at 49–50°C. In A–C, red cells were hydrolysed by bee venom phospholipase $A_2$ while in D, phospholipase $A_2$ from porcine pancreas was used. ( ● ), PC; (O), PE; (Δ), PS.

monolayer. This suggests that the asymmetric distribution of PS in the native red cell membrane does not result from a lack of transbilayer mobility but seems to be a consequence of interactions between PS and skeletal proteins. Although no direct demonstration of these interactions has yet been made in the native erythrocyte membrane, but spectrin and polypeptide 4·1 have been shown to interact preferentially with PS in mixed phospholipid vesicles and monolayers (Mombers *et al.*, 1979, 1980; Sato and Ohnishi, 1983; Bonnet and Begard, 1984). Additionally, it is known that the interactions between spectrin and PS are strengthened by the presence of PE in the lipid mixture (Mombers *et al.*, 1979).

Finally, it may be concluded that asymmetric transmembrane phospholipid organization in red cells is primarily maintained by the skeleton-membrane associations. As the asymmetric assembly of membrane phospholipids has already been established in various cell types (Op dem Kamp, 1979), it may be envisaged that this asymmetry in other cells is also maintained by similar mechanisms. This speculation

seems all the more plausible, since proteins similar to red cell skeletal proteins have been identified in the membrane-associated cytoskeleton of a variety of cells (Geiger, 1983; Cohen, 1983; Glenney and Glenney, 1983).

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