ERYTHROCYTE MEMBRANE

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ABSTRACT

In the sixties and seventies erythrocytes, or red blood cells (RBCs), have been extensively studied, much have been learned about this special cell particularly concerning its metabolism and gas transporter function. During the following years RBCs have lost their interest; however in this last decade with use of new approaches and the application of modern techniques, new data become again RBC in an interesting subject of study. Among the new data, one in particular is very poorly understood, I am referring to the adhesion molecules in RBC surface, that until know its presence was recognize and required in the other blood cells (such as neutrophils, lymphocytes, and platelets), or in endothelium cells.

In this paper I will talk about RBC membrane, its composition and organization, focus on its lipid and protein components and functions.

INTRODUCTION

Over the last 40 years red blood cells (RBCs) have been subject of many studies and articles. Much has been learnt from classical biochemical approaches about RBCs composition and membrane organization. RBCs have a characteristic that make this "simple" cell a unique model of study: ability to maintain its discoid shape and yet allowing cytoskeleton rearrangements that permit it to pass through capillaries. Mature RBCs in blood flow are the product of a differentiation process that starts in the bone marrow as hematopoietic stem cells differentiate to nucleate RBCs. After extrusion of nuclei and degradation of endoplasmic reticulum, reticulocytes emerge in the circulation, where they rapidly develop into mature RBCs 8-μm biconcave disk and with a 120 days life span[1]. During its life-time RBCs protein content and also lipid one, will be affected. RBCs suffer vesiculation processes however they can not synthesized *de novo* proteins or lipids so this leads to a different membrane composition during RBC life.

RBC membrane is composed by: 19.5% (w/w) of water, 39.5% of proteins, 35.1% of lipids and 5.8% of carbohydrates[2]. It has been identified by mass-spectrometry 340 different membrane proteins[1], being Band 3

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(B3) and glycophorin A (GPA) the two most abundant integral proteins of the red cell membrane, present in approximately 10^6 copies per cell[3,4]. RBC membrane has also lipid-rich domains usually called as lipid rafts or just rafts.

At the beginning RBCs were seen just as a gas transporter, they main function was to assure the transport of oxygen and carbon dioxide to and from tissues. Later RBCs adhesion was associated to functions such occlusion of sites of vascular interruption by platelets or as a preliminary stage in the migration of leukocytes out of blood stream and into tissues[4]. Accordingly, just in the last decade it has become clear that RBCs express on their surface many molecules known to provide adhesion functions in other cells[5,6,7]. Additionally RBCs are being recognized to have other functions, that 40 years ago were unthinkable, such the transport of iC3b/C3b-carrying immune complexes[1].

In this work I will talk about RBC membrane composition and organization, focus on its lipid and protein components and functions.

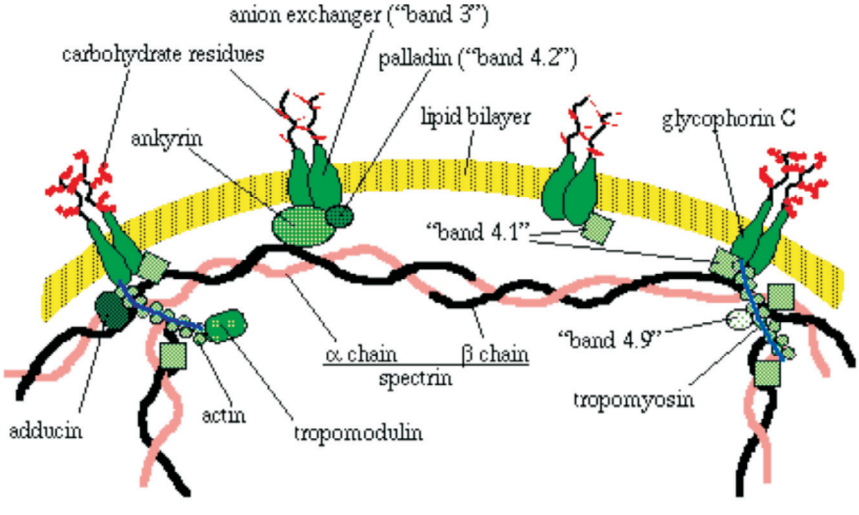

Figure 1 – Diagram of RBC membrane (http://www.ruf.rice.edu/~bioslabs/studies/sds-page/rbcmembrane.html)

RBC membrane lipids

RBC membrane is an asymmetric lipid bilayer and its membrane lipids composition is determinant to RBC shape and membrane fluidity[2].

The RBC membrane is composed by: 60% of phospholipids, essentially phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and phosphatidylserine (PS). It has also some phospholipidic minor components such as phosphatidylinositol (PI), PI-monophosphate (PIP), PI-4,5-bisphosphate (PIP_2), phosphatidic acid (PA), lysophosphatidylcholine (Lyso-PC) and lysophosphatidylethanolamine (Lyso-PE). Non-sterified cholesterol represents about 30% of the lipidic RBC compound, and the last 10% are glycolipids[2].

The asymmetry of the membranes plays an important role in the interactions between the cells and the outer environment. These RBCs have flippases and floppases so they can maintain the asymmetry by a flip-flop mechanism that is ATP-dependent.

In human RBC, PS and PE are located almost exclusively in the inner monolayer where PC and SM are minimally present. This arrangement is crucial to the cell homeostasis. For example, an exposure of PS in the outer surface leads to several mechanisms to achieve cell death, apoptosis[2].

Mature RBC cannot synthesize lipids *de novo*. Therefore these "simple" cells depend on lipid exchange and acylation of fatty acids, with plasma lipoproteins, as a mechanism for phospholipids repair and renewal[2].

LIPID-RICH MICRODOMAINS/ LIPID RAFTS

Lipid rafts are characterized by having a low density and being insoluble in cold nonionic detergents. They have several acronyms such as detergent-resistant membranes (DRM), triton-insoluble membranes (TIM) and triton-insoluble floating fractions (TIFF)[8].

Lipid rafts are membrane microdomains with a high level of organization given by its enrichment in cholesterol and glycosphingolipids (such as gangliosides (Gang) and sulfatides)[8,9] that contain saturated fatty acylchains[2,8]. Bulk plasma membrane contains less cholesterol, SM and Gang, and more phospholipids with unsaturated acyl chains. These components ensure a higher fluidity than rafts that have high level of organization and different composition as I said earlier[9].

Rafts cover over 4% of total RBC membrane proteins[10]. The major integral proteins in RBC rafts are stomatin, flotillin-1 and flotillin-2[2,11]. Duffy protein receptor and glycosylated phosphatidylinositol-anchored proteins (GPI-proteins: CD55, CD58, CD59) are also characteristic of lipid rafts[8]. Proteins that are associated to rafts become disassociated in the absence of cholesterol.

The size of lipid rafts has been studied in very different approaches that originate some conflicting results and interpretations. A conservative interpretation is that lipid rafts are probably structures with an average diameter in the range of 100nm to 200nm[9]. Not all the lipid rafts have identical protein or lipid composition, for example there is a subclass of these microdomains called caveolae that contains caveolin.

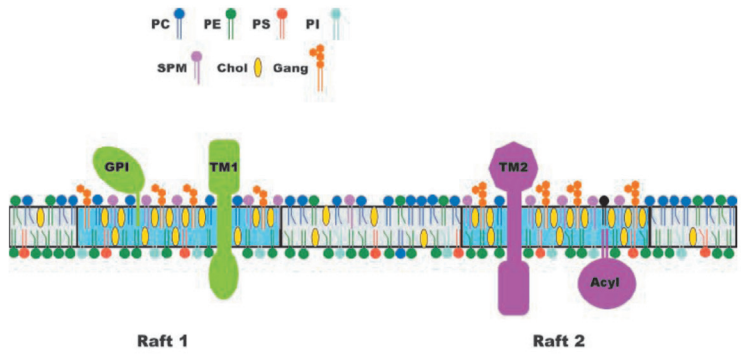

Figure 2 – Diagram of two different types of lipid rafts[8]

RBC MEMBRANE PROTEINS

Membrane proteins can be classified as "peripheral proteins" (for instance spectrin) when apparently are associated with only one face of the bilayer, or "integral proteins" (for example band 3) when they are strongly embedded into or through the lipid bilayer by hydrophobic domains within their amino acid sequences. Alternatively, membrane proteins can be grouped as (1) "cytoskeleton proteins" for those which belong to cytoskeleton, such as spectrin and actin; (2) "integral proteins" for those which are integrated in lipid bilayer, such as Band 3 (B3) and glycophorins (GPs); and (3) "anchoring proteins" for the ones that instance ankyrin and protein 4.2[2].

RBC membrane proteins are regulated by several post-translational mechanisms such as phosphorylation, methylation and glycosylation.

INTEGRAL PROTEINS

Band 3 (B3)

Protein B3 is the major integral protein present in RBC membrane

with 12-14 transmembrane (TM) segments[3], and there are about 10^6 copies per cell. It is a 911 amino acid multispanning membrane protein with molecular weight of 100KDa, also known as anion exchanger (AE1) that conducts bicarbonate-chloride exchange in RBCs[4].

Band 3 has an N-terminal cytoplasmic domain (amino acid 1-359) that can be associated to several proteins. This domain is responsible for the main membrane anchorage site to the cytoskeleton. Therefore it is very important for the flexibility and rigidity of RBC. The N-terminal domain is the site for binding of ankyrin, protein 4.2, and protein 4.1- membrane proteins responsible for the anchoring of membrane to RBC cytoskeleton- and also glycolytic enzymes and haemoglobin[2,3,13-20]. The B3 C-terminal domain (amino acid 360-911) carries out the anion exchange. It is formed by 12-14 TM segments with a short cytoplasmic tail (33 amino acids). Additionally it is responsible for the binding of carbonic anhydrase II[3,4,13-20]. B3 exists in RBC membrane as dimmers (70%), tetramers and higher order oligomers (30%). The tetrameric form binds basically protein 4.2 and ankyrin[4]. Altogether, B3 and respective associated proteins are known as the B3 macrocomplex. B3 macrocomplex is also related with glycophorin A and RhAG and/or Rh complex[4].

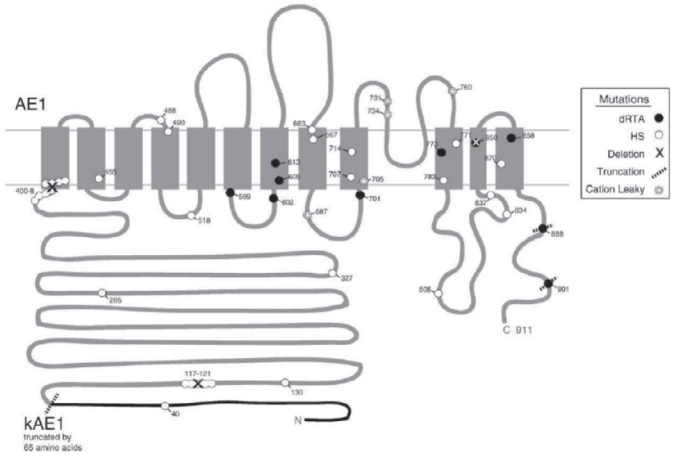

Figure 3 – Illustration of protein Band 3 and its possible mutations[4]

Band 3 configuration can be modulated by phosphorylation – by action of phosphotyrosine kinases (PTKs) like p53/56^{Lyn} – or dephosphorylation – by phosphotyrosine phosphatase (PTP). These reactions are responsible for example for the release of GEs to the cytoplasm and consequently activation of them[15,17,21,22].

Several B3 gene mutations have been already described such as B3 Coimbra, B3 Tuscaloosa, B3 Montefiore and B3 Fukuoka[2,3,23-25]. Several of these mutations affect RBC cytoskeleton interactions by altering the association point of B3 with ankyrin, protein 4.2 and/or protein 4.1. Others can lead to the reduction of B3 levels in RBC membrane affecting RBC functions in the human body[4,23-25]

Glycophorins: Glycophorin A (GPA)

Sialic acid residues are abundant in negatively charged RBC surface; this negative net charge is mostly from the sialic residues (60%) present in Glycophorin A (GPA), but also in other glycophorins (GPs), B3 and some glycolipids. These negative surface charges of RBC plays a crucial role in modulating RBC-RBC interactions and as well RBC interactions with vascular endothelium and other circulating blood cells[2,6].

Glycophorins or syaloglycoproteins are about 2% of total RBC membrane proteins[2,26] among there GPA is the major constituent repre-

senting 1.6% of total RBC membrane protein. Others such as GPB/E, similar to GPA, and GPC/D are present in lower amount. Interestingly GPC/D are non erythroid-specific.

Glycophorins have three domains[1], a cytoplasmic domain, which contains a cluster of basic residues that are located near the plasma membrane[2], a hydrophobic domain which exists as a single α-helix spanning the lipid bilayer, and[3] a extracellular domain which is heavily glycosilated[2].

GPA is a 131 amino acid syaloglycoprotein with an extracellular N-terminal. It has a single TM span and a cytosolic C-terminal tail. Nowadays, it is known that there is an important connection between GPA and B3 during their biosynthesis and trafficking to plasma membrane[2,4]. In fact B3 is critical for GPA synthesis and its stability, and GPA is not indispensable for B3 expression. This GP is associated to B3 in RBC membrane maybe so, it is present in similar amounts than B3 (10^6 copies per cell). The interaction between these two main integral proteins of RBC membrane originate the Wright (Wr^b) antigen[3,4]. The Wr^b antigen results from by the interaction of Glu^{658} B3 with a site or sites located either near the end of the extracellular domain of GPA or in the adjacent TM domain (TM8)[3,4]. Additionally antibodies to GPA decrease the lateral and rotational mobility of B3, and monoclonal antibodies of the Wr^b blood type antigen immunoprecipitates both B3 and GPA.

Total surface charge density is not affected in GPA-deficient red cells. However, they exhibit increased glycosylation of B3, probably due to the addition of excessive sialic acid, which should have been present on the GPA protein[2]. These cells maintain a normal RBC shape and deformability, thus suggesting that GPA may not be crucial to the RBC mechanical stability, deformability and shape change.

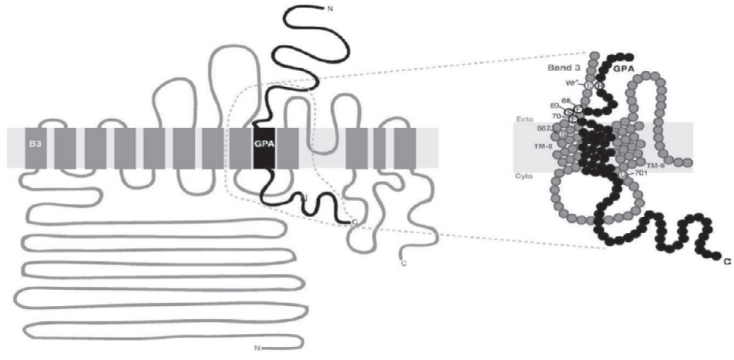

Figure 4 – Illustration of Band 3 and GPA interaction[4]

Blood Group Antigens

Human beings are mainly classified into distinct groups according to the ABO blood group system however other blood groups systems have been identified.

There are about 243 different determinants known in RBC membrane that belong to 19 distinct blood group systems.

Some of the more acknowledged are Rh blood group, P blood group, Lutheran blood group, Kell blood group, Lewis blood group, Puffy blood group, Kiddy blood group, LW blood group, li blood group and the Diego and Wright blood group antigens on B3. There are also some other minor blood group antigens such as Chido and the Rodgers blood group systems.

Glycosylphosphatidylinositol (GPI) Anchor Proteins

GPI anchors are glycolipid complex structures, highly conserved

with a common core region of a PI molecule to which are attached 4 sugars, one N-glucosamine and 3 mannoses.

Many membrane proteins are regulated by GPI anchor proteins such as (1) enzymes (for example acethylcholinesterase); (2) complementary defence proteins (DAF and CD55); (3) immunologic proteins; (4) receptors[2].

ANCHORING AND CYTOSKELETON PROTEINS

There are two major complexes responsible for the attachment of cytoskeleton network to the RBC membrane. In the first one, B3, as a membrane integral protein, binds to ankyrin/ protein 4.2 as establishing the interactions with the spectrin/actin complex. In the second one, has GPC/D, as membrane integral protein, binds to protein 4.1 complex and establish another interaction to spectrin/actin complex, connecting the RBC membrane to its cytoskeleton[2,16,18-20,27-34].

At this point we can assume that "peripheral proteins" can be subdivided into "anchoring" and "cytoskeleton" proteins.

Ankyrin is an anchoring protein and its major role is to create a tight association between spectrin and B3. The binding can be modulated by the extent of phosphorylation by the action of protein kinase A, casein kinase I or cyclic AMP-independent protein kinase. Protein 4.2 is also an anchoring protein that interacts with ankyrin and participates also in B3 macrocomplex structure. This protein has also an important role in the interaction between B3 macrocomplex and Rh complex by its connection with CD47[2].

Concerning the "cytoskeleton proteins" we have three foremost ones: spectin α and β, actin and protein 4.1. These three proteins form the "junctional complex"[2]. The erythrocyte membrane-cytoskeleton interactions can be modulated by phosphorylation of 4.1R by PKC, PKA, calmodulin or casein kinase I, which in turns leads to a decrease on the interactions between 4.1R-spectrin-actin and also 4.1R-Band 3 or 4.1R-GPC/D[35-38].

DISEASE STATES OF RED CELL MEMBRANE

Even nowadays in clinical haematology, RBC shape abnormalities are still widely utilized for first step-diagnoses before further detailed characterization of the phenotypes and genotypes of RBC membrane disorders. An interesting and functional hypothesis has been proposed that RBC membrane disorders can be regarded either as: (1) abnormalities in vertical interactions of membrane components, or (2) those in their horizontal interactions[2].

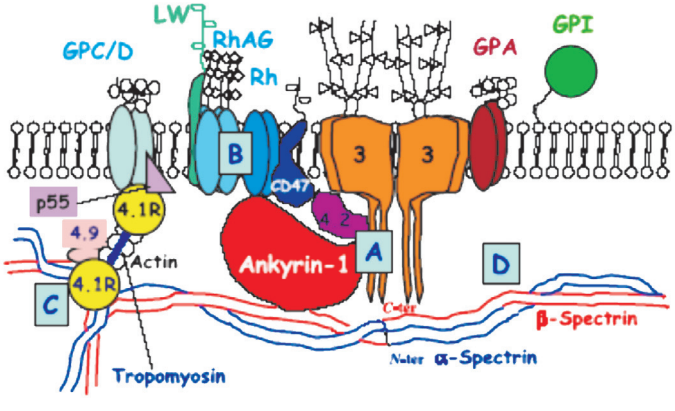

Figure 5 – Diagram of the two major points of interaction between RBC membrane and cytoskeleton. A: Band 3 macrocomplex; B: Rh complex C: Protein 4.1 complex[24]

An abnormality in vertical interactions occurs mainly in spectrin-ankyrin-B3 interactions, the protein 4.1-GPC linkage and also at the level of the interaction of skeletal proteins with membrane lipids (PE and PS mainly)[2]. The abnormalities in horizontal interactions usually concern either the self-association of spectrin to form its tetramers, which are the most important component of the cytoskeleton network or the interactions of this to protein 4.1 and/or actin[2].

Mutations in genes that codified proteins are the reason why these RBC membrane disorders occur, and are usually connected to several diseases[23-25].

The diseases that are associated to peripheral proteins are Hereditary Spherocytosis (HS), Hereditary Elliptocytosis (HE) and Southeast Asian Ovalocytosis (SAO)[23-25]]2].

For example a deficiency of spectrin occurs in HE and HS while a B3 disorder is associated with SAO or HS too. In Europe about 30% of the cases of HE has a deficiency in protein 4.1 levels in RBC membrane.

Sometimes a deficiency in a protein can lead to a secondary reduction of another one. This happens for instance with a rare type of HS, where the reduction or even absence of protein 4.2 are associated to the secondary decrease of CD47 levels, affecting the B3 macrocomplex-Rh complex interaction[23-25].

RBC ADHESION MOLECULES

RBCs are generally considered nonadhesive cells. However, several studies have reported the expression of a large number of adhesion molecules in RBC[1,5-7,39-48]. Adhesion molecules play a crucial role in cell-cell, and also cell-tissue, interactions and are also involved in a large range of biological functions such as erythropoiesis (differentiation, maturation, enucleation, and release of RBCs), self-recognition mechanisms, red cell turnover, and cell aging[6,39,40]. During this aging process some adhesion molecules are down-regulated and others are expressed at different stages and remain on mature RBCs (Table I)[39]

Mature RBCs express adhesion molecules for instance: CD44, CD47, CD58, LW/ICAM-4, and Lu[39]. Many of these adhesion proteins belong to

Table I – Adhesion molecules expressed by circulating erythrocytes[6]

Adhesion molecule and alternate name(s)	Ligand/adhesive function
Indian [*In(Lu)*-related p80, CD44]	Hyaluronan, possibly also fibronectin
Rh-related integrin-associated protein (IAP, CD47)	Thrombospondin
Lymphocyte-associated antigen-3 (LFA-3, CD58)	CD2
CD99, MIC2 gene product	Lymphocyte CD99 is necessary for formation of T-cell rosettes
JMH (semaphorin K1, SEMA7A, CD108)	Possible role in adhesion of activated lymphocytes
Ok[a](neurothelin, CD147)	Type IV collagen, fibronectin, laminin in other tissues
LW (ICAM-4, CD242)	Leukocyte integrins ($\alpha4\beta1$, $\alpha4\beta3$, $\alpha v\beta1$), platelet integrin ($\alpha IIb\beta3$), vascular integrin ($\alpha v\beta3$)
Lutheran (B-CAM/LU, CD239)	Laminin, possibly also integrins
Scianna (ERMAP)	Putative adhesive function
MER2 (CD151)	Forms laminin-binding complexes with integrins
CD36 (reticulocytes only), platelet glycoprotein IV, Nak[a] (platelets)	Thrombospondin (platelets)
VLA-4 (reticulocytes only), $\alpha4\beta1$ integrin (CD49d/CD29)	Thrombospondin, VCAM-1, fibronectin

the Immunoglobulin superfamily (IgSF) of proteins[40]. Normal RBCs do not adhere to circulating cells and vessel walls under normal circumstances, suggesting that RBC adhesion molecules could be inaccessible to their ligands. However RBCs become more adherently during for instance normal haemostatic conditions (clot formation), inflammation process, pathologic occlusion conditions and sickle cell disease[39-41,43].

The adhesion molecules that I will describe briefly support the idea that RBCs have at least the potential capability of adhering to a number of ligands including thrombospondin, fibrinonectin, laminin, hyaluronan, or to cells as endothelial cells and leukocytes.

LW/ICAM-4

LW is also known as ICAM-4 or CD242, this glycoprotein is an erythroid-specific membrane component that has a potencial role in adhesion or cell interaction events including hemostasis and thrombosis[5,6,39-42] ICAM-4 apparently is expressed as part of the Rh macrocomplex in RBC membrane[5,6].

ICAM-4 has several structural similarities to the ICAM family that has as primary cellular counter-receptor leukocyte specific β2 integrins[40]. However ICAM-4 is an unusual ICAM because it interacts with several types of integrins expressed on blood and endothelium cells[39,40,42]. This glycoprotein binds to CD11a/CD18 (LFA-1 or $\alpha_L\beta_2$ integrin), to CD11b/CD18 (Mac-1 or $\alpha_M\beta_2$ integrin), and to α_5 integrins on nonhemopoietic cells; $\alpha_4\beta_1$ on hemopoietic cells, and $\alpha_{IIb}\beta_3$ on platelets[40-42]. ICAM-4 as also been recently shown as a ligand for monocyte/macrophage-specific CD11c/CD18[40].

Selective binding of ICAM-4 to different integrins may be important for a variety of normal RBC function, and also relevant to the pathology of thrombotic events in sickle cell disease.

B-CAM/LU

B-CAM/LU was firstly described as a protein expressed at the basal surface of epithelial cells. This protein has a type-1 transmembrane glycoprotein containing five IgSF domains: two V-types and three C2-types[5,6,43].

B-CAM/LU is a laminin receptor relatively inactive in normal RBC surface although is highly expressed in sickle RBCs (SS RBCs) surface. The increase of adhesion to lamin in SS RBCs might be connected with the increase expression of B-CAM/LU in these cells[43].

CD47

CD47 is a 50KDa and highly glycosilated plasma membrane. It can also be call as integrin-associated protein (IAP) because its functions have been best studied in relation to integrin signalling. However now that is known that it can also interact with molecules and not just with integrins, CD47 seems to be a more appropriate name[47]. It is a transmembrane glycoprotein with five membrane spanning domains and a single extracellular V-type immunoglobulin super family (IgSF) domain.

This glycoprotein is associated with Rh macrocomplex and with cytoskeleton of intact RBCs, and it is functionally coupled to heterotrimeric Gi proteins, signal regulatory protein (SIRPα), and cholesterol[44]. CD47 is also a thrombospondin (TSP) family member receptor[5,6,44-47] and it can interact with several integrins of β_1, β_2, and β_3 families modulating platelet activation, cell motility and adhesion, and leukocytes adhesion, migration and phagocytosis[44]. CD47 cellular functions are well associated to the Ig domain that is required for the establishment of interactions with its associated integrins, its ligand TSP, and SIRPα[44-47].

In beginning CD47 was also associated to being a product of Rh gene because it was shown that in RBC Rh_{null} the CD47 levels decrease to even more low than usual. Although it is expressed in many different cells types in all tissues and in Rh_{null} individuals have normal levels of CD47 on cells other than RBCs[47].

In mature RBCs, with lack integrins, CD47 appears to mediate cell-cell interaction with SIRPα of splenic macrophages, to inhibit a phosphorylation cascade that blocks phagocytosis and prevents RBC clearance from the circulation[45].

As I said, CD47 also binds TSP and, in SS RBC, generate an intracellular signal that increases SS RBC adhesiveness that is associated to vaso-occlusive events[45-47]. CD47 SS RBCs structure is different from the structure of CD47 expressed in normal RBCs This structural change is responsible for the increase availability of SS RBCs adhere to endothelium using TSP as a mediator, although it levels are similar in the two types of RBCs[47]).

Sialyl Moieties

AS I said before, RBC membrane carries sialylated glycoproteins and glycoplipids. The sialic acid is negatively charged at physiological pH so it confers a halo negative charge that involves RBC that is called sometimes as zeta potencial. This charge generally help to prevent the interactions between RBCs and also with other blood cells, however now it appears that sialic acid residues, presented mainly in GPA, could facilitates cell-cell interaction of RBC.

Actually it was previously shown[48] that RBC are capable of interact, even with low affinity, with P-selectin, a integrin that contributes to the specificity of interactions among endothelial cells, platelets and leukocytes during inflammation, by a $Le^{a/b}$ antigen present in RBC surface. Matsui *et al.* have shown in this study the decrease of adherence of normal and SS RBC to P-selectin, using a P-selective blocking monoclonal antibodies or sialyl Lewis tetrasaccharide. In pre-treating RBCs with sialidase reduces their adherence to activated endothelial cells and immobilized recombinant P-selectin[48].

Although there are several more sialic acid rich structures in RBC membrane that could be involved in this process with a much higher affinity[5,6].

At the end we just can assure that a lot of work has to be made to try to understand this "simple" cell and the processes that she is involved, having always in mind that erythrocyte is not "just" a gas transporter.

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