4.1R-deficient human red blood cells have altered phosphatidylserine exposure pathways and are deficient in CD44 and CD47 glycoproteins

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ABSTRACT

Background

Protein 4.1R is an important component of the red cell membrane skeleton. It imparts structural integrity and has transmembrane signaling roles by direct interactions with transmembrane proteins and other membrane skeletal components, notably p55 and calmodulin.

Design and Methods

Spontaneous and ligation-induced phosphatidylserine exposure on erythrocytes from two patients with 4.1R deficiency were studied, using CD47 glycoprotein and glycophorin C as ligands. We also looked for protein abnormalities in the 4.1R - based multiprotein complex.

Results

Phosphatidylserine exposure was significantly increased in 4.1R-deficient erythrocytes obtained from the two different individuals when ligands to CD47 glycoprotein were bound. Spontaneous phosphatidylserine exposure was normal. 4.1R, glycophorin C and p55 were missing or sharply reduced. Furthermore there was an alteration or deficiency of CD47 glycoprotein and a lack of CD44 glycoprotein. Based on a recent study in 4.1R-deficient mice, we found that there are clear functional differences between interactions of human red cell 4.1R and its murine counterpart.

Conclusions

Glycophorin C is known to bind 4.1R, and we have defined previously that it also binds CD47. From our evidence, we suggest that 4.1R plays a role in the phosphatidylserine exposure signaling pathway that is of fundamental importance in red cell turnover. The linkage of CD44 to 4.1R may be relevant to this process.

Key words: phosphatidylserine, 4.1R deficiency, CD44, CD47, glycophorin C.

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Introduction

The red cell skeleton is critical for the mechanical properties of this cell. It is formed by a complex meshwork of proteins which imparts a great degree of elasticity.[1,2] Protein 4.1, hereafter referred to as 4.1R, is a major protein of the skeleton. It consists of four functional domains. The N-terminal 30 kDa domain binds glycophorin C (GPC), p55 (membrane-associated guanylate kinase homologs),[3-7] band 3,[8,9] and calmodulin.[10] The *EPB41* gene encodes 4.1R. *EPB41* has at least two initiator codons. In erythroid precursors, only the downstream initiator codon is used, leading to an 80kDa 4.1R isoform.

CD47 (integrin-associated protein, IAP) is a 47-52 kDa membrane protein with an amino-terminal IgV domain, a multiple-membrane-spanning region and different carboxyl-terminal cytoplasmic domains generated by alternative splicing.[11-13] CD47 is part of the Rhesus (Rh) subcomplex within the band 3-based multiprotein complex.[14,15] It is much reduced in regulator type Rh_{null} patients.[16] It is also secondarily reduced in hereditary spherocytosis associated with missing protein 4.2[15,17] or band 3.[14] CD47 binds the carboxyl-terminal cell-binding domain of thrombospondin-1 (TSP-1)[18-20] and also the agonist peptide 4N1K derived from this domain. TSP-1 is an adhesive molecule produced predominantly by platelets, and is known to be involved in the vaso-occlusive crises associated with sickle cell disease.[18] Known cellular ligands for CD47 on other cell types include macrophage SIRP-α:[21,22] this interaction is thought to be important in self-recognition mediated by CD47.[23] No extracellular ligands are known for GPC. As mentioned above, CD47 forms part of the Rh-band 3 supercomplex of the human erythrocyte membrane which may function to regulate CO_2 and bicarbonate transport.[24-26] CD47 is substantially diminished in p4.2-deficient erythrocytes, which are also deficient in major components of the Rh complex, thus it is likely that CD47 interacts directly with protein 4.2 in human erythrocyte membranes, which does not appear to be the case in mice.[15,17] The Rh-band 3 complex includes the $RhAG_2$-Rh protein trimer,[27,28] CD47, ICAM-4 and band 3 dimers/tetramers.[29,30]

Red cell turnover accounts for the highly regulated processing of approximately 10^{12} effete red cells per day. This is governed by a process termed eryptosis,[31] which has several functional differences to apoptosis. Phosphatidylserine (PS) exposure on the surface of the extracellular membrane leaflet appears a pivotal event in the initial stages of eryptosis. Ligation of CD47 using monoclonal antibody BRIC 126 and 4N1K peptide-mediated PS exposure on red cells is associated with a loss of viability *in vitro*.[32] Ligation of GPC (with mouse monoclonal antibody BRIC-10) also caused PS exposure and demonstrated similar effects. Notably though, this effect was cancelled with mutant forms of GPC, missing exon 2 (Yus phenotype) or exon 3 (Gerbich phenotype) in the *GYPC* gene,[33] but was unchanged with elongated GPC variant Ls^a (duplication of exon 3).[33] These observations suggested that both GPC and CD47 participate in signaling pathways that singly or in concert result in the extracellular exposure of PS on the red cell surface.

It seemed interesting to investigate spontaneous and ligation-induced PS exposure in 4.1R(-) red cells lacking 4.1R, especially since 4.1R is a PS binding protein.[34] We investigated the erythrocytes from two patients: (i) patient A, described before,[35] with a homozygous mutation, ATG>AGG, which abolishes the downstream initiator codon and (ii) patient B, presenting with severe 4.1(-) ellipto-poikilocytosis and a homozygous mutation that has been incompletely elucidated so far (*Baklouti and Morinière, unpublished data*).

In both patients, spontaneous PS exposure was normal, although there was a slightly higher background of PS exposure in patient B which may be related to the quality of the red cells. PS exposure was decreased upon ligation of GPC and increased upon ligation of CD47. However, missing 4.1R caused the absence, reduction or alteration of GPC and CD47, and other components, so that the significance of the variations in PS exposure does not have an obvious explanation. We looked for other protein abnormalities in the 4.1R-based multiprotein complex. 4.1R itself, GPC and p55 were missing or sharply reduced, as shown before in patient A[36,37] and confirmed in patient B. There was a change in the distribution of CD47 isoforms (patient A) or a sharp reduction of this protein (patient B). This is interesting because CD47 is currently known to belong to the band 3-based multiprotein complex. CD44 was missing, a fact not reported before and locating CD44 within the 4.1R-based multiprotein complex. CD44 is known to have significant physiological roles in inflammation and binds to hyaluronan of the extracellular matrix. It has recently been concluded that CD44 acts as the erythrocyte ligand on binding to hyaluronan and induces tethering and rolling on a synthetic substrate in shear condition.[38] Thus CD44 may play a role in red cell turnover, and its linkage to 4.1R may be relevant to this process.

Design and Methods

Patients

Patient A's case has been reported before.[35] Briefly, this man was born in north-western Spain from parents who were first cousins. He presented with the rare form of 4.1(-) ellipto-poikilocytosis. He underwent splenectomy and cholecystectomy in 1979. He did not need blood transfusions thereafter. He carries a mutation, ATG→AGG in the homozygous state, which cancels the downstream initiator codon, the only remaining initiator codon in the erythroid line. The corresponding allele is designated as 4.1 R(-) Madrid.

Patient B is a child who was born in 2004. This child also presented with severe 4.1(-) ellipto-poikilocytosis (red blood cell count: 1.85×10^{12}/L; hemoglobin: 5 g/dL; reticulocytes: 215×10^{9}/L). His monthly transfusion need led to subtotal splenectomy by the age of 1 year. The transfusion requirements became occasional (red blood cell count: 3.13×10^{12}/L; hemoglobin: 8.6 g/dL; reticulocytes: 463×10^{9}/L), often made necessary by viral infections. Missing 4.1R could have been unveiled following

subtotal splenectomy, the intervals between transfusions having become longer. Removal of the spleen stump is currently under discussion. The parents were first cousins and showed a typical 4.1R (-) trait as described earlier.[39,40] Using sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE), 4.1R was found to be reduced by 32% and 30% in the father and the mother, respectively. From the beginning, the child was considered homozygous for a null allele, which is designated 4.1R (-) Troyes. To date, the genomic mutation has only been partially characterized. It consists of a deletion within the *EPB41* gene that appears in the homozygous state (*Baklouti and Morinière, unpublished data*).

Blood from patients A and B was collected and shipped at ice water temperature. Informed consent to the studies conducted was obtained from patient A and the parents of patient B.

Specific monoclonal antibodies

The specific antibodies utilized are shown in Table 1.

Flow cytometry for detection of receptor-mediated phosphatidylserine exposure

Erythrocytes were washed twice in Hanks' balanced salt solution (HBSS) pH 7.4 (Invitrogen, Paisley, UK) and once in HEPES buffer (10 mM HEPES, pH 7.4, 140 mM NaCl) (Sigma-Aldrich, Poole, UK).

Cells were diluted in HEPES buffer to a concentration of approximately 4×10^6 cells/mL. They were then incubated with 10 μg/mL of each monoclonal antibody, 100 μg/mL anti-CD47 peptide or non-active anti-CD47 peptide (Table 1), both synthetic, in 200 μL of HEPES in 96-well plates (Sigma-Aldrich). Negative controls were treated in the presence of HEPES buffer only. Cells were incubated at 37°C for 18 h with gentle agitation, and were then washed with HBSS. Next, 1x binding buffer and 5 μL of fluorescein isothiocyanate-labeled annexin (BD Biosciences, Oxford, UK) were added to each well. The plate was gently vortexed and incubated at room temperature in the dark for 15 min. Samples were added to fluorescence-activated cell-sorting (FACS) tubes containing 300 μL 1x binding buffer. Tubes were placed on ice and assayed for PS exposure using a FACScan Vantage SE flow cytometer (Becton Dickinson, Oxford, UK). Data were obtained using CellQuest software v7.5.3 (Becton Dickinson, Oxford, UK).

Protein immunoblotting and staining

Erythrocyte ghosts were prepared as previously described.[41] Proteins were run on 4-12% Bis-Tris SDS-PAGE gels in NuPage MES/SDS running buffer (Invitrogen, Paisley, UK). One dimensional PAGE gels in the presence of SDS were stained with SYPRO Ruby (Bio-Rad, Hemel-Hempstead, UK), as described by the manufacturer, to visualize membrane proteins. For immunoblotting, samples were transferred to polyvinyldifluoride membranes (Bio-Rad) using a wet blot mini trans-blot cell at 4°C overnight (Bio-Rad). They were immunoblotted with monoclonal antibodies and/or polyclonal antibodies and then horseradish peroxidase- conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added for detection. Antibody binding was detected by chemiluminescence using an ECL Plus kit (GE Healthcare, Little Chalfont, UK) followed by subsequent development onto autoradiography Hyperfilm-ECL (GE Healthcare).

Blood group genotyping using BLOODchip

Genomic DNA was extracted from peripheral blood leukocytes from the control and both patients A and B using the QIAamp DNA Blood Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. DNA quality control thresholds were set at a minimum concentration of 40 ng/μL and an A260/A280 ratio of between 1.6 and 1.95. BLOODchip multiplex polymerase chain reactions (PCR) (Progenika Biopharma, S.A., Derio, Spain) were carried out using

Table 1. Origin of specific monoclonal antibodies and synthetic peptides.

Target	Specific antibody of reagent	Characteristics or origin
4.1R	Anti 4.1R synthetic peptide (residues 241-262): NH_2-(C)EHLNLLEEDYFGLAIWDNATSK-COOH	Coupled to keyhole limpet hemocyanin via *N*-terminal cysteine residues as previously described[55]
55	Anti p55 synthetic peptide (residues 439-455): NH_2-GVDETLKKLQEAFDQAC-COOH)	*Ibid*
GPC	Anti-GPC monoclonal antibody BRIC 10	International Blood Group Reference Laboratory, National Blood Service, Bristol, UK
CD47	Anti-CD47 monoclonal antibody BRIC 126	*Ibid*
CD47	Anti-CD47 peptide (TSP-1 mimetic peptide) 4N1K (NH_2-KRFYVVMWKK-COOH)	University of Bristol
CD47	Non-active anti-CD47 peptide 4NGG (NH_2-KRFYGGMWKK-COOH)	*Ibid*
Band 3	Anti-band 3 monoclonal antibody BRIC 155	International Blood Group Reference Laboratory, National Blood Service, Bristol, UK
GPA	Anti-GPA monoclonal antibody BRIC 256	*Ibid*
CD44	Anti- CD44 monoclonal antibody BRIC 235	*Ibid*

primers to amplify the genomic regions of interest carrying the 116 blood group-defining single nucleotide polymorphisms (SNP) for the following blood group systems: ABO, RH, KEL, JK, FY, MNS, CO, DO and DI as described by Avent *et al.*[42] The PCR products were amplified in a uniform manner by the inclusion of multiplex-amplifiable probe hybridization tag binding sites on the 5' ends of most of the primers.[43] The technical approach adopted by allele-specific hybridization DNA Array Methodology has been described by Tejedor *et al.*[44] The amplified PCR products were fragmented, labeled and hybridized against an array (BLOODchip) composed of probes corresponding to sequences within the amplified SNP (40 probes per SNP). The scoring of each blood group-specific SNP, either homozygous or heterozygous, was established by BLOODchip proprietary software (Progenika Biopharma), which analyzes the fluorescence from the pairs of probes complementary to each SNP. Groups of SNP were also analyzed to establish a predicted phenotype for each blood group system.

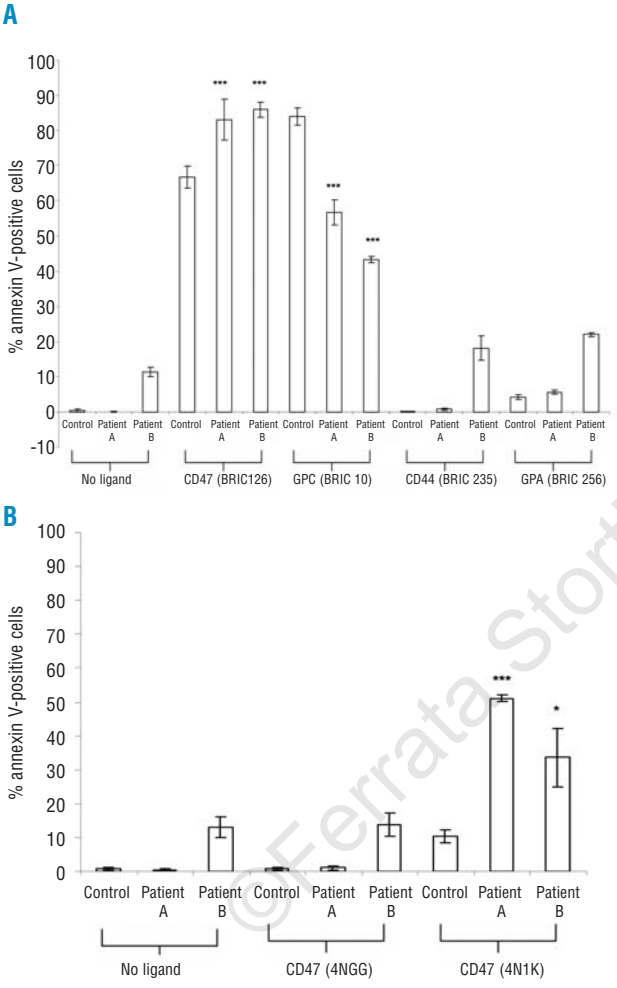


Figure 1. Flow cytometry of erythrocytes from 4.1R (-) patients A and B and controls following ligation with natural and synthetic CD47 and GPC ligands and resultant PS exposure, detected by annexin V binding. Erythrocytes were incubated with ligands as described in the Design and Methods section, and treated with annexin V-fluorescein isothiocyanate (FITC). Mean percentages of annexin V-FITC positive cells are shown with respect to each ligand. Spontaneous PS exposure was not statistically different in patients A and B, despite the significant protein deficiencies, from that in normal controls. However, the exposure was relatively higher in patient B in all experiments. This might be a transport or preparation artifact. (A) Ligation of CD44 had no effect. Ligation of CD47 (monoclonal antibody BRIC 126) resulted in increased PS exposure with anti-CD47. Ligation of GPA had no effect. Ligation of GPC triggered less PS exposure than in the normal control. (B) Ligation with synthetic peptide CD47 ligand (4N1K) and control peptide (4NGG) produced a similar effect to that seen with monoclonal anti-CD47 ligands. Each experiment was repeated three times, and statistical analysis was performed using a paired Student's *t* test. The statistical significance is indicated on the figure as follows **p*<0.05, ***p*<0.01, ****p*<0.0005.

Results

Receptor-mediated exposure of phosphatidylserine in 4.1R (-) red cells

Experiments were conducted using a single collection of red cells from patients A and B, with patient A having an age- and travel- matched control, and patient B having a control whose blood sample was collected on the same day. All experiments, unless otherwise stated, were repeated a minimum of three times, and statistical analysis was performed following flow cytometry using Graphpad™ software.

For some unknown reason, patient B's red cells showed some degree of spontaneous PS exposure in the absence of ligands, contrasting with patient A's and control red cells (Figure 1). Ligation of GPC markedly increased PS exposure in the control, as previously recorded,[33] and did so, but to a lesser extent, in patients A and B (still less in patient B than in patient A) (Figure 1). Ligation of CD47 markedly increased PS exposure in the control, as previously observed,[32] and did so to an even greater extent in patients A and B (Figure 1). Ligation of CD44 and GPA produced no particular effect with regard to the control (except that patient B's red cells, as already pointed out, showed spontaneously increased PS exposure) (Figure 1).

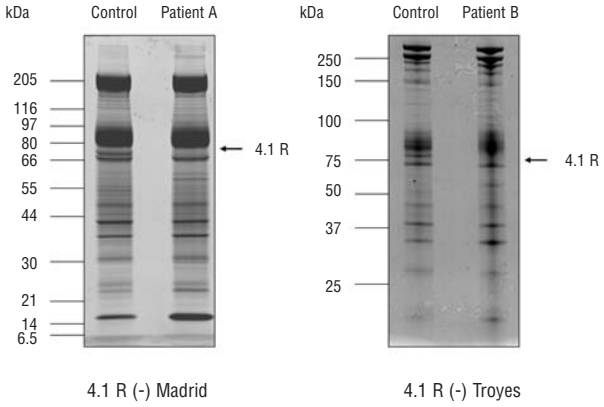
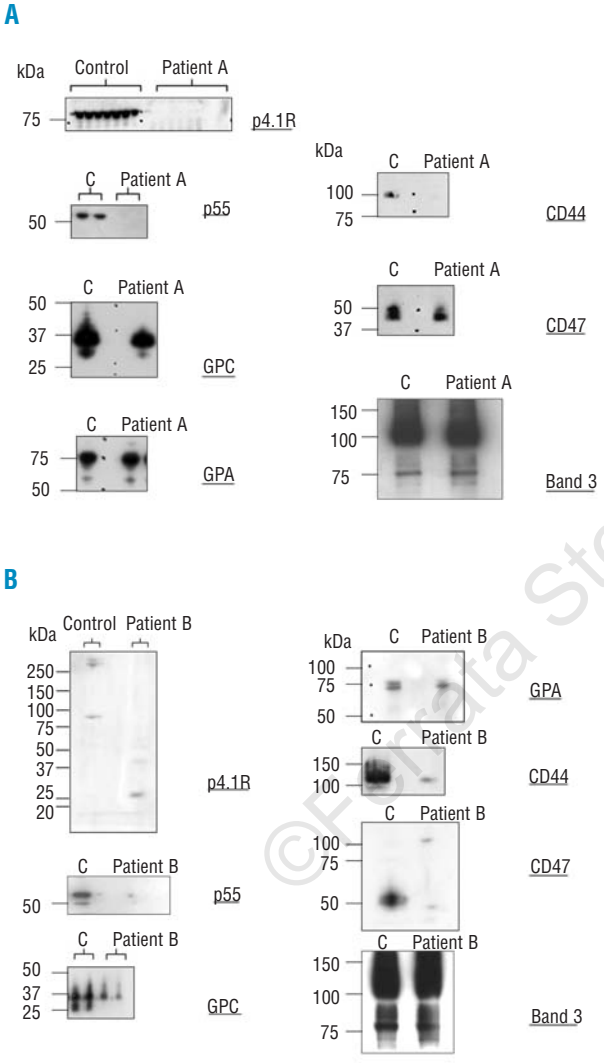


Figure 2. Sypro Ruby stained SDS-PAGE gels of red cell membrane from 4.1R (-) patients A and B, and from controls. The absence of 4.1R is clearly visible in samples from patients A and B and is labeled with an arrow.

The protein composition of the 4.1R-based multiprotein complex in the patients

Patients A and B showed an absence of 4.1R, as previously observed[35] (Figure 2). Patient A also showed a marked reduction in GPC and absence of p55 (Figure 3), confirming previous results.[37] Patient B showed the same changes, except that p55 was very faintly present rather than absent (Figure 3). Patient A had a reduction of high molecular weight isoforms of CD47 (Figure 3). CD47 has five known spliceoforms in the 47-52 kDa region.[45,46] CD47 expression in patient B was very low (Figure 3). In both patients A and B, CD44, a glycoprotein that may be involved in the traffic of erythroid cells from the bone marrow, and expresses Indian (IN) blood group antigens,[47] was sharply diminished, a not previously reported finding in 4.1R-deficient patients. The amounts of band 3 and glycophorin A (GPA) appeared normal (GPA was slightly diminished in patient B) (Figure 3). Altogether, the protein phenotypes were highly consistent in patients A and B. Table 2 presents a summary of altered protein expression in both human and murine 4.1R deficient red cells.



Figures 3. Western blotting of red cell membranes from 4.1R-deficient patients A and B and age- matched controls with various anti-red cell membrane and membrane skeletal antibodies. The following antibodies were used: anti-4.1R (polyclonal antibody); anti-p55 (rabbit polyclonal antibody); anti-GPA (murine monoclonal antibody BRIC 256); anti-GPC (BRIC 10); CD47 (BRIC 126); anti-band 3 (BRIC 155); CD44 (BRIC 235). Multiple lanes were loaded for anti-4.1R and anti-p55 blots. (A) Patient A. The most salient findings were the absence of 4.1R and of p55, the reduction in GPC, the absence of the high molecular weight isoforms of CD47, and the absence of CD44. (B) Patient B. The most salient findings were the absence of 4.1R and near total absence of p55, the reduction in GPC, the absence of CD47, and the near complete absence of CD44.

Blood group antigen expression on 4.1R(-) erythrocytes

Blood group antigen expression on the surface of the 4.1R-deficient red cells was determined using serological and genomic investigations. The expression of most blood group antigens appeared normal and genotypes were consistent with a *European descent*, with one exception (Table 3). Patient A's sample was typed by serology as Fy (a-b-) which is very rare in Europeans but common in West Africans.[48] Subsequent molecular investigations, using allele-specific primers[49,50] and the blood group genotyping system BLOODchip,[42,51] demonstrated that patient A has the rare *FY*0/FY*X* genotype which predicts weakened Fy^b antigen expression on erythrocytes. This weakened antigen expression, coupled with hemizygosity for the *FY*B* allele, made serological detection of the Fy^b antigen very difficult and could have led to the erroneous interpretation that the Duffy antigen was diminished, as is the case in the 4.1R(-) mouse.[52] Taken together, patients A and B showed no blood group abnormality that could have been related, directly or indirectly, to missing 4.1R.

Table 2. Status of relevant proteins in 4.1R(-) human and mice.

Protein	4.1R(-) (humans)	4.1R(-) (mice)
4.1R	▼▼	▼▼
GPC	▼	▼▼
p55	▼▼	▼▼
Actin	N.D.	▼
Tropomyosin	N.D.	▲
Tropomodulin	N.D.	→
Dematin	N.D.	→
Adducin	N.D.	▲
CD44	▼	N.D.
AQP1	N.D.	→
Spectrin	N.D.	→
Ankyrin	N.D.	→
Band 3	→	▼
GPA	→ (A) ▼ (B)	▲
NHE1	N.D.	▲

▼▼: *significantly downregulated or absent.* ▼: *downregulated.* →: *expressed at identical levels.* ▲: *upregulated; N.D: not defined.*

Table 3. Blood group phenotyping and genotyping in patients A and B.

Blood group	PATIENT A Serology	PATIENT A Genotype	PATIENT B Genotype
ABO	ND	ND	O1O1
RHD	ND	ND	RHD negative
RHCE	ND	ND	ccee
KEL	K+k+	Kk KP*B/KP*B JS*B/JS*B	kk KP*B/KP*B JS*B/JS*B
JK	JK(a+b-)	JK*A/JK*A	JK*A/JK*A
FY	Fy(a-b-)	FY*0/FY*X	FY*A/FY*B
MNS	M+N-S-s+	MMss	MNSs
DI	ND	DI*B/DI*B	DI*B/DI*B
DO	ND	DO*B/DO*B	DO*A/DO*B
CO	ND	CO*A/CO*A	CO*A/CO*A

DNA samples were processed using the blood group genotyping system BLOODchip. ND : Not determined.

Discussion

Receptor-mediated exposure of phosphatidylserine in 4.1R(-) red cells

In normal controls, ligation of CD44, lying within the 4.1R-based multiprotein complex, failed to produce any change in PS exposure. The ligation of GPA failed to do so as well, which is not surprising given its location away from the 4.1R-based multiprotein complex. The results of PS exposure in the patients are to be interpreted in the light of the protein content of the 4.1R-based multiprotein complex. The primary absence of 4.1R triggered the secondary absence, reduction or alteration of GPC, p55, CD47 and CD44. Such a dramatic change did not modify the spontaneous PS exposure even though 4.1R is a PS-binding protein.[34] 4.1R is in contact with the inner leaflet of the lipid bilayer and must interact with the internal PS molecules. One would have assumed, therefore, that untethered PS molecules would more easily move into the outer leaflet. If this assumption is true, one must further hypothesize that the compensating mechanism, allowing the return of PS molecules to the internal leaflet, increases its transport capacity. One may ask why ligands could have some effects on CD47 or GPC, which are absent, reduced or altered, notwithstanding the other abnormalities in the protein composition of the 4.1R-based multiprotein complex. We were somewhat surprised to find that reduced levels of CD47 would be expected to result in a reduced level of PS exposure following ligation. It is possible, therefore, that the absence of 4.1R indicates that it plays a key role in the regulation of PS exposure moderated by CD47. More simply, 4.1R, being a PS-binding protein,[34] would directly oppose PS exposure under normal conditions.

The proteins missing in the 4.1R-based multiprotein complex in humans

In the 4.1R-deficient mouse, Salomao *et al.*[52] showed extensive loss of the skeletal meshwork and the presence of bare areas of the membrane. Band 3, and proteins that are bound to it, were increased or present in normal amounts. On the other hand, Rh proteins, as well as XK and Duffy proteins, were much reduced. GPC was missing. The amount of p55 appeared to be normal. Salamao *et al.*[52] postulated that, besides the band 3 (tetramers)-based multiprotein complex, another complex existed, the 4.1R-based multiprotein complex. A fraction of band 3 (dimers) would be present in the 4.1R-based multiprotein complex.

In humans, a 4.1R-based multiprotein complex has not been defined with such accuracy. There are no reasons why it should be the exact replica of that in the mouse (Table 2). The absence of 4.1R and of p55, and the pronounced reduction of GPC, first recorded in patient A,[36,37] were confirmed in patient B. Other proteins (CD47 and CD44) were found to be missing, reduced or altered.

So far, CD47 has been recognized to belong to the band 3-based multiprotein complex, specifically in the Rh sub-complex.[15,17] CD47 was reduced in Rh_{null} patients.[16] In patient A, CD47 was reduced at the expense of its high molecular weight isoforms, and was almost completely missing in patient B. There are at least five different isoforms of CD47 known to exist in humans, and the pattern found in patient A may be consistent with 4.1R interacting specifically with CD47 isoforms with longer C-terminal domains.[45,46] An interaction between CD47 and 4.1R has been suggested before[53,54] (Plummer *et al., unpublished data*). These findings further strengthen the view that CD47 might belong to both the band 3-based and the 4.1R-based multiprotein complexes. However, its detailed interactions within the latter complex are yet to be defined. Incidentally, protein 4.2 is normal in 4.1(-) patients and we also found its expression was normal.[35]

The marked reduction of CD44 in both 4.1(-) patients has never been reported before. It strongly indicates that CD44 belongs to the 4.1R-based multiprotein complex. This is consistent with previous studies that identified, reciprocally, binding sites for 4.1R on the *C*-terminal domain of red cell CD44.[10]

No changes in either patient were found in the studied blood groups antigens, including the Rh, Kell, and Duffy blood groups. The absence of Duffy blood group reactivity in patient A could have been mistaken for a genuine absence, and thus might have hinted at a resemblance with the missing Duffy proteins in 4.1R (-/-) mice.[52] However, it resulted only from a rare genotype in this patients.

Altogether, the 4.1R-GPC-p55 triad was missing *en bloc* and must account for the core (or a core) within the 4.1R-based multiprotein complex. This situation was also found in mice. In humans, our results indicate that CD44 and CD47 may also belong to this complex. The absence of 4.1R hampers their insertion within the 4.1R-based multiprotein complex. The case of CD47 is of particular interest since it is already known to belong to the band 3-based multiprotein complex.

Authorship and Disclosures

KPJ, ZEP, DJH, TEM, KLS, AW and JRS all performed experiments, prepared figures and the manuscript. FG and JD obtained samples, planned and designed experiments, prepared and reviewed the manuscript. NDA designed the experiments planned and drafted the manuscript. All authors proof read the final version of the manuscript.

NDA is a consultant for Progenika Biopharma, and JRS is a consultant for Progenika US, the suppliers of BLOODchip. The other authors had no potential conflicts of interest.

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