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Lack of Protein 4.1a in Red Blood Cells of the Hereditarily Anemic Belgrade Laboratory (*b/b*) Rat

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Abstract We have demonstrated that the red blood cell (RBC) membrane of the hereditarily anemic Belgrade laboratory (*b/b*) rat contains protein 4.1b isoform, only. The evidence are given that the synthesis of protein 4.1 in the *b/b* rat reticulocytes is the same as in normal rat. When haemolytic anaemia was induced in normal rat by in vivo phenyhydrazine treatment the same phenomenon, i.e., the absence of protein 4.1a in the RBC membrane was observed. The increase of 4.1a isoform was monitored in RBCs during the recovery of normal rat after phenyhydrazine treatment. Hence, the portion of membrane protein 4.1a isoform is increasing during rat RBC aging. Likewise, when the RBC life span is prolonged (but not normalised) in the *b/b* rats by iron-dextran treatment protein 4.1a is present in small portion in the RBC membrane. All these data indicate that the lack of protein 4.1a isoform in the *b/b* rat is due to the presence of young RBCs in the circulation. J. Cell. Biochem. 75:56–63, 1999.

Key words: RBC membrane; protein 4.1; RBC aging; *b/b* rat

The Belgrade laboratory (*b / b*) rat suffers from hereditary haemolytic, microcytic, hypochromic anaemia inherited as an autosomal trait [Sladić-Simić et al., 1963, 1966]. The anaemia is accompanied by decreased amount of haemoglobins and globins in red blood cells (RBCs) [Sladic-Simic et al., 1969; Popovic et al., 1993], disbalanced and reduced globin synthesis [Marjanovic et al., 1994; Zarić et al., 1998b,c]. The genetic defect is manifested at the level of iron release within cell [Edwards et al., 1980; Garrick et al., 1993; Bowen et al., 1987; Faircich et al., 1992]. Recently, *b* mutation has been attributed to a missense mutation in the gene encoding the putative iron transporter protein Nramp2 [Flemming et al., 1998].

The b/b rat RBCs display anisocytosis, poikilocytosis, and have a shortened half-life [Sladic-Simić et al., 1966, 1969]. We have previously demonstrated that a hsp70-like protein is specifically targeted to the b/b rat RBC membrane [Zarić et al., 1998a]. As hsp70 proteins act as "quality control system" [Hammond et al., 1995], we presumed that hsp70-like protein is bound to the malfolded (mutated) protein in the b/b rat RBC membrane [Zarić et al., 1998a].

The mammalian RBC membrane skeleton consists of a flexible protein framework that determines cell membrane shape, deformability, and structural integrity [Bennett et al., 1993; Benz Jr. et al., 1993]. Protein 4.1 is a major component of the red blood cell skeleton that laminates the inner surface of the plasma membrane [for review see Lux et al., 1995; Delaunay et al., 1995; Conboy, 1993]. It contributes to the skeleton anchoring to the membrane through interactions with band 3 [Jons et al., 1992; Hemming et al., 1995], glycophorin C, p55 [Hemming et al., 1995; Marfatia et al., 1995; Alloisio et al., 1993], and tightens the interaction of spectrin and actin [Horne et al., 1993; Schischmanoff et al., 1995; Discher et al., 1995; Lorenzo et al., 1994]. The importance of interaction involving protein 4.1 is emphasized by the association of protein 4.1 deficiency and defective binding of protein 4.1 to spectrin [for review see Delaunay, 1995; Lux et al., 1995] with various types of haemolytic anaemia.

As the b/b rat RBCs are defective [Sladić-Simić et al., 1966, 1969], we were interested to study the status and synthesis of protein 4.1. The b/b rat suffers from hereditary severe hae-

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molytic anaemia. Therefore, we have compared the data obtained on this mutant with another model for haemolytic anaemia, anaemia induced in normal rat by in vivo phenylhydrazine treatment. The defect in protein 4.1 was the same in both mutant (*b/b*) and phenylhydrazine treated rat, i.e., the absence of 4.1a isoform was observed. Further analysis revealed that the lack of 4.1a is due the presence of young RBCs in both animals.

MATERIALS AND METHODS

Animals

Laboratory rats of Wistar strain (3-month-old) were used in all experiments. Belgrade laboratory (*b/b*) rats were obtained from the original Belgrade colony [Sladić-Simić et al., 1969]. The animals were fed a standard laboratory rat diet. The iron treatment of b/b rats was performed as described previously [Biljanovic-Paunović et al., 1992] Reticulocytosis was induced in normal (+/+) rats by phenylhydrazine (PHZ) as described previously [Bowen et al., 1987]. Reticucytosis was also induced by the removal of 2% body weight of blood by heart puncture. The bled normal rats were sacrificed 3 days later.

Unless otherwise indicated, the experiments outlined below were performed a minimum of three times and yielded similar results. Representative experiments are shown.

Preparation of Red Blood Cell Membranes

Haemoglobin-depleted red blood cell (RBC) membranes (ghosts) were prepared as described by Dodge et al. [1963], using 5 mM sodium phosphate pH 8.0, containing EDTA (1 mM) and PMSF (0.03 mM) as the lysis buffer [Bodine IV et al., 1984]. The membranes were collected by centrifugation at 10,000*g* for 20 min and washed with the same buffer three to four times. The first supernatant (cytosol) was carefully collected for isolation and further investigation of cytoplasmic proteins. All operations were carried out at 0–4°C. The protein concentration was determined by the method of Lowry et al. [1951] with bovine serum albumin as a standard.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE according to the buffer systems of Leammli using 10% SDS polyacrylamide gels [Laemmli, 1970]. The gels were stained in Coomassie Brilliant Blue R-250 [Sambrook et al., 1989].

Antibodies

Anti protein 4.1 polyclonal antibodies were kindly provided by Dr. D. Drenckhahn, University of Würzburg. These antibodies were raised in rabbits against human RBC protein 4.1. Dr. D. Drenckhahn demonstrated that the antibodies can also recognise rat RBC protein 4.1 [Jons et al., 1992]. These antibodies were used for immunoblotting in 1: 400 dilution.

Anti protein 4.1 polyclonal antibodies used for immunoprecipitation studies were a kind gift from dr Jennifer Pinder, King's College London. These antibodies were also raised in rabbits against human RBC protein 4.1, and were used in 1:50 dilution in immunoprecipitation studies.

Immunoblotting

Immunoblotting was performed using both 10% SDS-PAGE and wet electrophoretic transfer of proteins from gel to nitrocellulose filters (Hybond C, Amersham, Arlington Heights, IL) [Burnette, 1981]. The bound immunoglobulins were visualised using alkaline phosphatase-labelled goat anti-rabbit IgG and NBT and BCIP as substrates (Promega, Madison, WI).

In Vitro Cell Labelling

10^8 of reticulocytes were incubated in 1 ml of modified Eagle medium (MEM) without Methionine (Torlak, Belgrade), 1% foetal calf serum (Gibco, Grand Island, NY), and [^{35}S]-Methionine (50 µCi/mL; 1,000 Ci/mmol; Amersham SJ1015) for 3 h at 37°C in 5% CO_2 in air [Bodine IV, 1984]. Cells were then pelleted, washed twice in cold PBS, and membrane and cytosolic fractions were subsequently isolated as indicated above.

Immunoprecipitation Studies

Fractions of labelled cell membranes were diluted with 1 volume of immunoprecipitation buffer (150 mM NaCl/10 mM Tris-Cl (pH 7.2)/1% Triton X-100/1% NP-40/0.1% SDS/10 mM EDTA/10 mM EGTA), and were incubated with anti protein 4.1 antibodies for 1 h at 4°C. After adding a 10% Protein A Sepharose (Protein A Sepharose CL-4B, Pharmacia Biotech, Gaithersburg, MD) in incubation buffer, the suspension

was incubated for another hour at 4°C. The beads were washed four times with immunoprecipitation buffer, than suspended in SDS-sample buffer, and boiled for 2 min [Harlow et al., 1988; Maede et al., 1988].

RESULTS

The *b/b* Rat RBC Membrane Lacks Protein 4.1a Isoform

When the RBC membrane and membrane skeleton proteins of the *b/b* rat were separated by SDS-PAGE and compared with the normal rat RBC membrane the differences in the region of protein 4.1 were observed (Fig. 1A). The protein 4.1 was then identified by immunoblotting using the polyclonal antibodies raised against the human 4.1 (see Materials and Methods). Both isoforms of protein 4.1 (4.1a and 4.1b) were present in the normal rat RBC membrane (Fig. 1B, lane +/+). However, the complete absence of protein 4.1a isoform was detected in the *b/b* rat RBC membrane (Fig. 1B, lane *b/b*).

Since the *b/b* rat is characterised by reticulocytosis (30% of reticulocytes) the other control used in this study was the normal rat in which the reticulocytosis (20% of reticulocytes) was induced by blood removal (bleeding; Fig. 1A, lane bled). The slight decrease in protein 4.1a isoform portion was detected in the RBC membrane of the bleeding rat, Fig. 1B, lane bled.

The haemolytic component in the *b/b* rat anaemia is very severe and we were interested to determine the status of protein 4.1 isoforms in another case of haemolytic anaemia. Phenyhydrazine treated normal rat (PHZ rat) is a good model for study of defects in severe haemolytic anaemia (Fig. 1A, lane PHZ). When we looked for protein 4.1 isoforms the very same defect as in the *b/b* rat RBC membrane, i.e., the lack of protein 4.1a, was detected in the PHZ treated rat RBC membrane (Fig. 1B, lane PHZ).

The *b/b* Rat RBC Soluble Protein 4.1

The protein 4.1a might be present in the b/b rat RBC soluble fraction (cytosol) but for some reason it is not incorporated into the membrane. To address this question we have analysed the *b/b* rat RBC cytosol for the presence of protein 4.1. These analysis revealed the total lack of protein 4.1a in the *b/b* rat RBC cytosol (Fig. 2, lane *b/b*) while both protein 4.1 isoforms were present in the normal and bleeding rat RBC cytosol (Fig. 2, lanes +/+ and bled, respectively). The RBC cytosol in the PHZ treated normal rat contained protein 4.1b isoform, only (Fig. 2, lane PHZ). Hence, again the

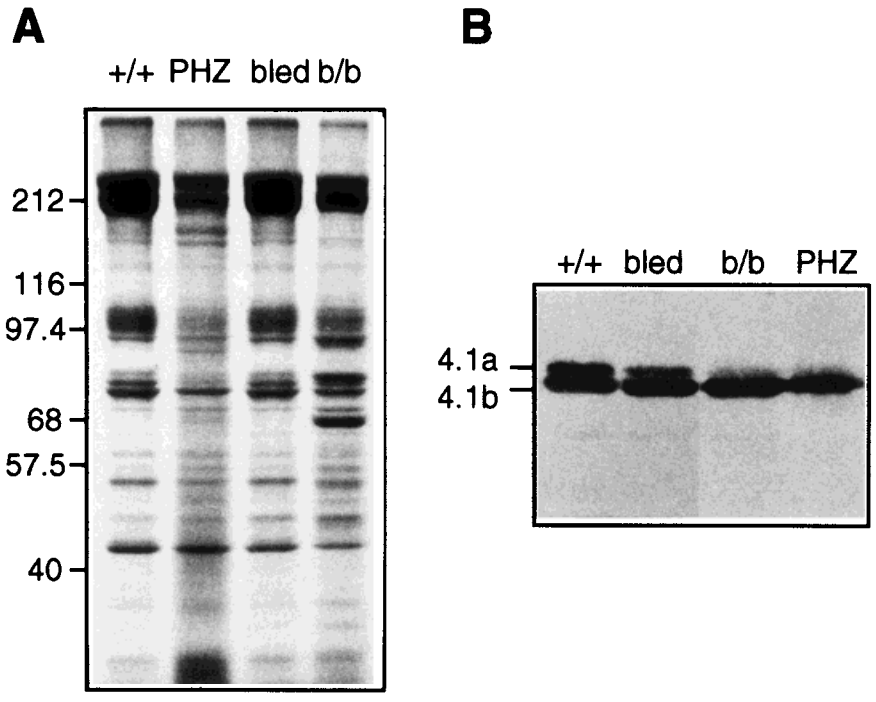

Fig. 1. Protein 4.1a is not present in the b/b RBC membrane. **A**: SDS-PAGE analysis of RBC membrane isolated from: normal (lane +/+), PHZ-treated normal rat (lane PHZ), normal after blood removal (lane bled) and b/b rat. Ninety µg of each sample was loaded on 10% SDS-polyacrylamide gel and Coomassie Brilliant Blue stained after the electrophoresis. Molecular weight markers (Sigma, St. Louis, MO) are indicated. **B**: Immunoblot analysis of rat RBC membrane proteins isolated from different animals, as indicated in the figure. Twenty µg of each sample was separated on 10% SDS gel, transferred to nitrocellulose membrane and developed using anti protein 4.1 polyclonal antibodies, diluted 1:400.

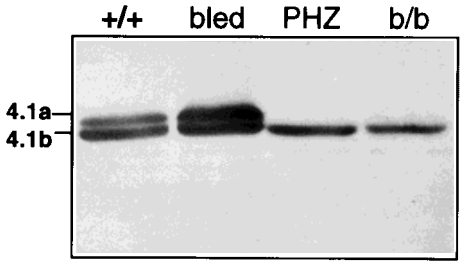

Fig. 2. The b/b rat RBC cytosol contains no protein 4.1a isoform. RBC cytosolic fractions (20 µg each) isolated from different animals, as indicated in the figure, were separated on 10% SDS gel, transferred to nitrocellulose membrane, and developed using anti protein 4.1 polyclonal antibodies, diluted 1:400.

b/b and PHZ treated rat RBCs displayed the same defect.

One Protein 4.1 Isoform is Synthesised in Rat Reticulocytes

Next, the synthesis of protein 4.1 in the *b/b* and PHZ treated rat reticulocytes was analysed. The reticulocytes were in vitro labelled for 3 h with [^{35}S]-Methionine and the membrane and cytoplasmic fractions were then isolated. The newly synthesised protein 4.1 incorporated into the reticulocyte membrane was subsequently identified by immunoprecipitation (see Materials and Methods). In all animals analysed (the *b/b*, PHZ and bleeding rat) reticulocyte membrane contained one newly synthesised protein 4.1 isoform, only (Fig. 3). According to the relative molecular mass of this radioactive band (it comigrated with 4.1b in Coomassie Brilliant blue stained gel) we have concluded that 4.1b isoform was synthesised and associated with the rat reticulocyte membrane. It should be noted that the same protein 4.1 isoform was detected in reticulocyte soluble fraction in all animals analysed (data not shown).

Protein 4.1a Isoform Level Increases During RBC Aging

The data presented so far suggest that the *b/b* and PHZ treated rats display the same defect at protein 4.1 level. Since both rats suffer from severe haemolytic anaemia the absence of protein 4.1a could be attributed to the young RBCs. If this is the case, then the aging of RBCs after the PHZ treatment should be followed by the increase of protein 4.1a portion. The normal rat was treated with PHZ and subsequently left to recover. RBC membrane proteins were then isolated from the same animal 2, 9, 16, and 23 days after the PHZ treatment. Immunoblotting analysis revealed that protein 4.1a first appeared in the RBC membrane 9 days after the PHZ treatment (Fig. 4, lane 3). The relative ratio of protein 4.1a to 4.1b was nearly normalised (compare with the untreated rat, Figure 4, lane +/+) 23 days after the PHZ treatment (Fig. 4, lane 23d). These data suggested that 4.1a isoform will appear in the membrane of the rat mature and aging RBCs.

Finally, we wanted to prove that in the b/b the lack of protein 4.1a was due to the premature RBC death, too. It is well known that the iron-dextran treatment improves all the haematological parameters of the b/b rat [Biljanović-Paunovic et al., 1992]. More importantly, the haemolysis is decreased and the RBC life-span is prolonged [Sladić-Simić et al., 1972]. Indeed, we have detected the small portion of protein 4.1a isoform in the RBC membrane of the iron-dextran treated *b/b* rat (Fig. 5, lane +Fe). The small increase in portion of protein 4.1a was expected because the RBC life-span in the b/b rat even after iron-dextran treatment is not normalised (Sladić-Simić et al., 1972].

DISCUSSION

In this study we present evidence that the lack of protein 4.1a in the *b/b* rat RBC membrane is due to the presence of young RBCs. First, we have demonstrated that only one protein 4.1 isoform is synthesised in both normal and *b/b* rat reticulocytes and that it is most probably 4.1b isoform. Protein 4.1a was absent in the RBC membrane of the normal rat in which haemolytic anaemia was induced by phenyhydrazine treatment. However, protein 4.1a slowly appeared in the membrane during RBC aging in rats after phenyhydrazine treatment. Likewise, when RBC life span was prolonged in the b/b rat by in vivo iron treatment, protein 4.1 was detectable in the RBC membrane. Therefore, all these data indicated that protein 4.1a increases during rat RBC aging.

The mammalian RBC membrane protein 4.1 exists in two major electrophoretic isoforms: 4.1a and 4.1b [Leto et al., 1984]. Diverse protein 4.1 isoforms (erythroid and nonerythroid) are the result of the tissue- and developmentally-regulated alternative splicing of pre-mRNA transcribed from a single gene, as demonstrated in human [Conboy et al., 1991;

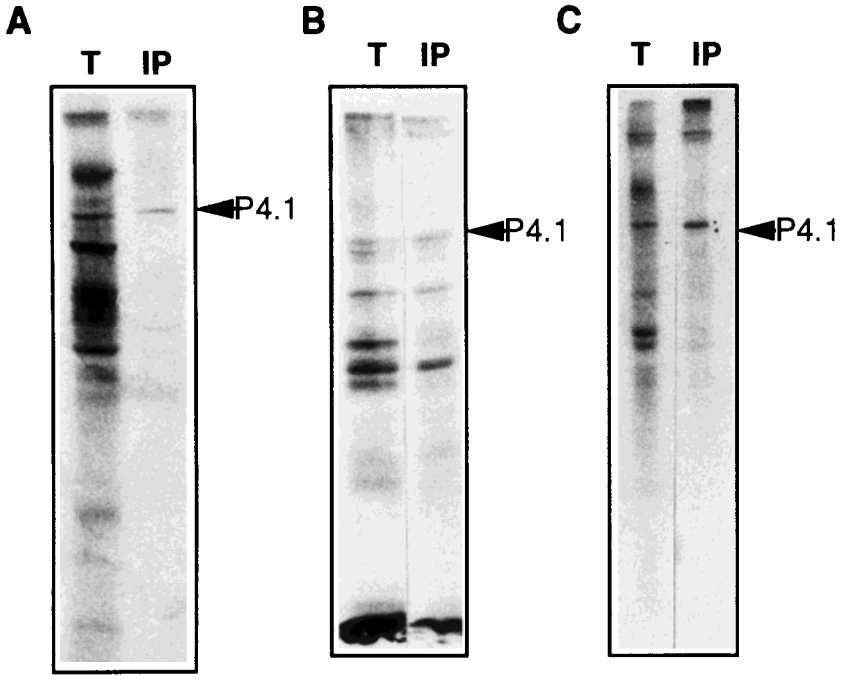

Fig. 3. The synthesis of protein 4.1 in b/b (A), bled (B), and PHZ-treated (C) rat reticulocytes. **A**: **Lane T:** Total newly synthesised membrane proteins isolated from b/b rat reticulocytes were subjected to SDS-PAGE and subsequently processed for autoradiography, **lane IP:** immunoprecipitate of newly synthesised protein 4.1 using polyclonal antibodies diluted 1:50. **B**: The same as in A, except that the bled rat reticulocyte membrane proteins **(lane T)** and protein 4.1 **(lane IP)** were analysed. **C**: The same as in A, except that the PHZ-treated +/+ rat reticulocyte membrane proteins **(lane T)** and protein 4.1 **(lane IP)** were analysed.

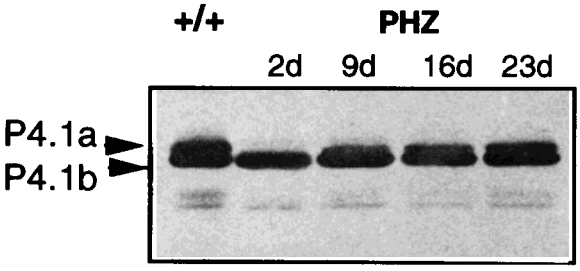

Fig. 4. The appearance of protein 4.1a during the recovery of normal rat RBCs after PHZ treatment. Immunoblot analysis of rat RBC membrane proteins isolated from normal **(lane +/+)** and normal rat treated with PHZ (RBC proteins were isolated from the same animal 2, 9, 16, and 23 days after PHZ treatment, lanes 2d, 9d, 16d, and 23d, respectively). Twenty µg of each sample was separated on 10% SDS gel, transferred to nitrocellulose membrane, and developed using anti protein 4.1 polyclonal antibodies, diluted 1:400.

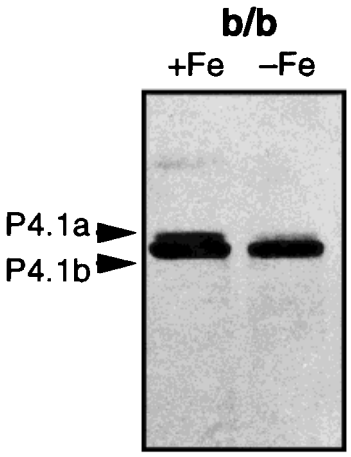

Fig. 5. Protein 4.1a is present in the iron-treated b/b rat RBC membrane. Immunoblot analysis of rat RBC membrane proteins isolated from: iron-dextran treated b/b rat (**lane +Fe**) and non-treated b/b rat (**lane −Fe**). Twenty µg of each sample was separated on 10% SDS gel, transferred to nitrocellulose membrane, and developed using anti-protein 4.1 polyclonal antibodies, diluted 1:400.

Baklouti et al., 1997] and mouse [Huang et al., 1993]. We could not find any literature data on rat protein 4.1 gene expression, except in the case of some nonerythroid tissues [Baklouti et al., 1997]. Therefore, we have performed some preliminary RT-PCR analysis of rat reticulocyte protein 4.1 mRNA. Primers were synthesised to amplify the erythroid specific 10kD spectrin/actin domain according to the mouse protein 4.1 genomic sequence [Huang et al., 1993]. One isoform of rat protein 4.1 mRNA was detect in rat reticulocyte by RT-PCR and high homology with mouse gene was observed (data not shown). Furthermore, no differences in the splicing of the 10kD spectrin/actin domain of protein 4.1 mRNA was observed between normal and b/b rat (data not shown).

It has been demonstrated that only protein 4.1b isoform is synthesised in mouse erythroid cells [Maede et al., 1988; Hanspal et al., 1992]. Furthermore, there are reports indicating that young RBCs contain 4.1b predominantly, while

4.1a is the major form in old cells [Muller et al., 1987]. We, as well, have determined that protein 4.1b is the only isoform synthesised in both normal and b/b rat reticulocytes.

Posttranslational modification, i.e., deamidation of Asn^{502} is responsible for conversion of protein 4.1b to 4.1a, as demonstrated for human protein 4.1. [Inaba et al., 1992a]. Under physiological conditions, deamidation of Asn in proteins and peptides occurs mostly in time-dependent manner by proceeding through a five-membered succinimide ring intermediate and it appears to be the major pathway of spontaneous structural damage in cellular proteins and it can readily be detected by changes in the electrophoretic mobility of the protein) [Geiger et al., 1987]. Since the conversion of protein 4.1b to 4.1a, under physiological conditions, occurs in a time-dependent manner, it has been demonstrated that deamidation is an excellent marker for RBC aging [Inaba et al., 1992a].

The mammalian RBC has an exquisite internal clock which allows the cell to circulate for extended periods of time with apparently full functional capabilities and then to be quickly and reliably removed from the circulation. Normal aged erythrocytes are selectively removed from the circulation via phagocytosis by the reticuloendothelial system [Clark, 1988; Kosower, 1993]. During its life span, the erythrocytes are exposed to various insults and undergoes physical and chemical changes that increase with cell age. Changes are observed in cell volume, in cell density, and in cytoplasmic and membrane components. The aged erythrocytes seem to have a normal ratio of α to β spectrin and normal contents of ankyrin and band 4.1 [Suzuki et al., 1989]. However, several changes in RBC membrane proteins have been reported to occur during the aging process, such as irreversible cross-linking between spectrin and haemoglobin, polymerisation of membrane proteins, and oxidative damages [Kosower, 1993; Suzuki et al., 1989]. The most obvious alternation in membrane proteins during RBC aging is an increase in the ratio of protein 4.1a to 4.1b, as demonstrated in mouse [Muller et al., 1987] and human [Sauberman et al., 1979]. Thus, it seems that the 4.1a to 4.1b ratio is a useful index for red cell age. Furthermore, the 4.1a/4.1b ratio in various mammals was established to be closely related to the life span of the RBCs and that the conversion of 4.1b into 4.1a very slowly progresses as red blood cells age [Maede et al., 1988]

Based on these literature data we have hypothesised that the *b/b* rat RBC membrane contains only protein 4.1b because young cells are constantly being produced. We have proven this hypothesis in two ways. Firstly, we have analysed protein 4.1 from young RBCs of the normal rat after phenyhydrazine treatment. Phenylhydrazine is known to cause accelerated senescence of erythrocytes and often used to replace the circulation mature erythrocytes with a population of new RBCs for experimental studies [Suzuki et al., 1989]. No protein 4.1a was present in neither RBC membrane nor cytosol in rat immediately after phenyhydrazine treatment. The recovery of the treatment was followed by an increase of RBC membrane protein 4.1a isoform. Secondly, we have demonstrated that protein 4.1a can be synthesised (formed) in the b/b rat when haemolysis was suppressed by iron-dextran treatment. In summary, our data suggest that protein 4.1b to 4.1a conversion during RBC aging takes place in rat, too, as previously documented for human [Inaba et al., 1992a], mouse [Muller et al., 1987], and canine [Inaba et al., 1992b].

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