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Correlation between protein 4.1a/4.1b ratio and erythrocyte life span

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Erythrocyte membranes from various healthy mammals contained a doublet of protein 4.1a and 4.1b, which appeared to differ by 2–3 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The ratio of protein 4.1a/4.1b showed much variety among animal species, and the 4.1a/4.1b ratio correlated to the mean erythrocyte life span, that is, the mean cell age in circulating blood. We also found that the 4.1b is the predominant form in the immature erythroid cells such as reticulocytes and K562 cells. In addition, the 4.1b but not 4.1a protein was metabolically labeled with [^{35}S]methionine in the erythropoietic cells from anemic mouse. Immunological detection showed that there is a doublet of minor variants of protein 4.1 with apparent molecular masses slightly more than those of 4.1a and 4.1b. The ratio of these minor isoforms designated as 4.1a+ and 4.1b+ revealed the alteration during erythrocyte senescence as observed in 4.1a/4.1b ratio. These results show that protein 4.1 may be synthesized as 4.1b and 4.1b+ and intercalated into membrane skeletons at an early stage of erythroidal differentiation, and that the posttranslational modification into 4.1a and 4.1.a+ appears to occur by a common mechanism in many mammalian species. Feline erythrocytes, however, appeared to lack such a postsynthetic processing of protein 4.1, and exhibited one major component of 4.1b with the other minor variant of 4.1b+.

Introduction

A network of skeletal proteins based on spectrin and actin lines the cytoplasmic side of the mammalian erythrocyte membrane, and contributes to maintaining cellular integrity and elasticity [1]. Protein 4.1 is thought to strengthen this network by forming a stable ternary complex with spectrin and actin [2,3] and by anchoring these skeletal proteins to the lipid bilayer [4] or to intrinsic membrane proteins such as band 3 [5] and glycophorin [6]. The importance of interaction involving protein 4.1 is emphasized by the association of protein 4.1 deficiency [7] and defective binding of protein 4.1 to spectrin [8] with various types of hemolytic anemia.

Human erythrocyte protein 4.1 can be resolved into two polypeptides on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) that have been designated as 4.1a and 4.1b [9], which appear to differ by 2 kDa in the carboxy-terminal domain [10]. These two proteins are closely related in their sequence and function [11]. It appears that 4.1a is probably produced by posttranslational modification, since young red cells contain 4.1b predominantly, while 4.1a is the major form in old cells [12]. Recently, Mueller et

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; MEM, minimal essential medium.

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al. [13] reported, using a serial hypertransfusion technique, that the most obvious alteration in membrane protein during in vivo mouse red cell aging was an increase in the ratio of protein 4.1a/4.1b. Thus, it seems that the 4.1a/4.1b ratio is a useful index of red cell age. In the present study, we report on the protein 4.1a/4.1b ratio in erythrocytes from various mammals and its close relation to the life span of their cells, the mean cell age in circulating blood, and on how the conversion of 4.1b into 4.1a very slowly progresses as red cells age. It is also demonstrated that the erythropoietic spleen cells from anemic mouse could synthesize only protein 4.1b, supporting the post-translational alteration of protein 4.1b into 4.1a.

Experimental procedures

Preparation of cellular membranes. Erythrocytes were collected from healthy adult cows (Holstein), horses (thoroughbred), sheep (Merino and Suffolk), goats (Saanen and Tokara), pigs (Large-white-Landrace), rabbits (New Zealand white), mice (BALB/c and ICR), dogs (mongrel), cats (domestic) and human subjects. Animals were housed at the Faculty of Veterinary Medicine of Hokkaido University.

Heparinized blood was filtered through an α-cellulose/microcrystalline cellulose column to remove leukocytes and platelets. Filtered cells were washed three times with phosphate-buffered saline (PBS). Washed cells were lysed with 40 vols. of lysing solution (5 mM Tris-Cl/1 mM EDTA/0.8 mM PMSF, pH 7.8) and centrifuged for 15 min at 18000 × *g*. Membranes were washed twice with the same solution, then once with 250 mM sucrose/0.2 mM PMSF/1 mM EDTA-Tris (pH 7.4). The resultant membranes were suspended in the same sucrose solution and stored at −80°C. Prolonged washing of the membranes with the lysing solution described above caused no alteration of the protein 4.1a/4.1b ratio in human, dog and goat cells. In the case of these cells at least, our procedure for preparation of the membranes preserved the values of the protein 4.1a/4.1b ratio in the ghosts, which were the same values as those from the whole red cells (data not shown) as reported in mice [13]. In addition, we observed no preferential loss of 4.1b which was reported to occur in the course of membrane preparation [12].

Membranes from K562 cells were prepared as previously reported [14].

Antibody production. Proteins 4.1a and 4.1b were prepared from canine erythrocytes according to the method of Tyler et al. [15]. After DEAE-cellulose chromatography, the preparation was subjected to preparative SDS-PAGE, because it was slightly contaminated with other peptides. The protein was excised from the gel and concentrated. Protein 4.1 (200 μg) was emulsified with an equal volume of Freund's complete adjuvant and injected into a New Zealand white rabbit twice with an interval of 3 weeks. 4 weeks later, a booster injection (100 μg) was given intravenously, and the antiserum was obtained 4 days subsequently. Anti-protein 4.1 IgGs were purified by affinity chromatography on a Protein A-Sepharose CL-4B column.

Labeling of erythropoietic spleen cells. Erythropoietic spleen cells were prepared from female ICR mice as described by Braell and Lodish [16].

The erythroid cells were washed twice in methionine-free minimal essential medium (MEM, Flow Laboratories), resuspended at a 10% concentration in methionine-free MEM supplemented with 5% dialyzed fetal calf serum, and incubated at 37°C for 20 min. Cells were then labeled with [^{35}S]methionine (200 μCi/ml, 1000 Ci/mmol, New England Nuclear) at 37°C. For pulse-chase experiments, further incorporation of [^{35}S]methionine was stopped by the addition of unlabeled methionine (0.5 mM) and chased for 15–180 min at 37°C. After labeling, cells were washed three times with PBS at 4°C. Washed cells were lysed in 4 vols. of 150 mM NaCl/20 mM Tris-Cl (pH 7.5)/1 mM EDTA/1 mM PMSF/1% Triton X-100. The lysates were centrifuged for 10 min at 15000 × *g*. The supernatants were removed, the pellets were washed once with the same buffer and reconstituted to the original volume. Solid urea and 2-mercaptoethanol were added to each fraction to give a final concentration of 9 M and 5%, respectively.

Immunoprecipitation. Samples were diluted with 9 vols. of immunoprecipitation buffer (150 mM NaCl/20 mM Tris-Cl (pH 7.5)/1% Triton X-100/1% sodium deoxychorate/0.1% SDS/2 mM

EDTA/2 mM EGTA), and anti-4.1 IgGs (5 μg) were added. After incubation for 3 h at 4°C, 20 μl of a suspension (50%, v/v) of Protein A-Sepharose beads were added and incubated for 1 h at 4°C. The beads were washed four times with the immunoprecipitation buffer, then suspended in SDS-PAGE sample buffer [17] and boiled for 1 min.

SDS-PAGE and immunoblotting. SDS-PAGE was performed as previously described [14]. Quantitative densitometric scanning of the gels stained with Coomassie brilliant blue was carried out using a densitometer (Helena Laboratories).

The gels containing proteins labeled with [^{35}S]methionine were processed for fluorography with EN^{3}HANCE (NEN), dried and exposed to Kodak XAR-5 film with intensifying screens (DuPont) at −80°C.

Immunological detection of protein 4.1 and immunologically related polypeptides was carried out as described in our recent report [14] using anti-4.1 IgGs.

Results

Protein 4.1 in various mammalian erythrocytes

The compositions of erythrocyte membrane proteins were similar in various mammals, including human. Fig. 1A shows the patterns of three major membrane proteins, designated as bands 3, 4.1 and 4.2 (nomenclature of Steck [18]) on a Coomassie brilliant blue-stained SDS-polyacrylamide gel. Proteins 4.1a and 4.1b in membranes subjected to SDS-PAGE were identified by immunoblotting with anti-protein 4.1 IgGs raised against protein 4.1 prepared from canine erythrocytes. From such immunoblots in erythrocyte membranes from many species of mammals – human, dog, sheep, goat, cow, horse, pig, mouse and rabbit *, two polypeptides were immunospecifically visualized with high intensities (Fig. 1B, lanes 1–9). The corresponding doublet polypeptides were clearly observed on a Coomassie blue-stained gel (Fig. 1A, lanes 1–9, indicated by open circles). These two polypeptides in each mammalian cell appear to differ by 2–3 kDa. Their apparent molecular weights ranged from 77000 to 84000 on SDS-PAGE (Table I). Based on their mobility on SDS-PAGE, specific immunorecognition with anti-4.1 IgGs, and the relative abundance among the immunoreactive polypeptides, we concluded that these protein doublets were proteins 4.1a and 4.1b in the erythrocytes from each of the mammals used.

A significant difference was observed for protein 4.1 in feline erythrocyte membranes. Feline erythrocyte membranes showed a single band of 4.1 with a molecular mass of 77 kDa on SDS-PAGE and immunoblotting, as shown in Fig. 1A, B (lane 10). Increasing the amount of protein subjected to SDS-PAGE enabled visualization of another 84 kDa polypeptide recognized with anti-4.1 (Fig. 1B, lane 10′). Similar results were obtained for individual preparations from membranes from six healthy adult cats.

In any case, the extent of heterogeneity in mammalian erythrocyte protein 4.1 seems to be

TABLE I

PROTEIN 4.1a/4.1b RATIO IN ERYTHROCYTES FROM VARIOUS MAMMALS

Molecular weights were calculated on SDS-polyacrylamide gels using molecular marker proteins (Bio-Rad) as standards. Data for 4.1a/4.1b are expressed as means ± S.D. or mean values.

Subjects	Molecular weight ($\times 10^{-3}$)		4.1a/4.1b	*n*
	4.1a	4.1b		
Human	81	79	1.52 ± 0.23	(4)
Dog	81	78	1.27 ± 0.13	(6)
Sheep	84	81	1.67 ± 0.14	(5)
Goat	81	78	1.58 ± 0.18	(5)
Cow	80.5	77.5	1.57 ± 0.19	(5)
Horse	81	78	1.50 ± 0.19	(4)
Pig	79	77	1.03 ± 0.12	(5)
Mouse	79	77	0.41 ± 0.04	(4)
Rabbit	81	78	0.31	(2)
Cat [a]		77	–	(6)

[a] Only a single major band (4.1b) was detected (see text).

* There is no definitive answer to the question of why the antibody generated in the rabbit bound to protein 4.1 in rabbit erythrocyte membranes. It appears to be significant that SDS denaturation of canine protein 4.1 used as an antigen to produce the antiserum in the rabbit (Experimental procedures) or of protein 4.1 in the rabbit erythrocyte membranes subjected to SDS-PAGE and immunoblotting (Fig. 1, lane 9), or both, caused the changes of antigenic determinants in protein 4.1. A similar phenomenon has been reported by Granger and Lazarides [19].

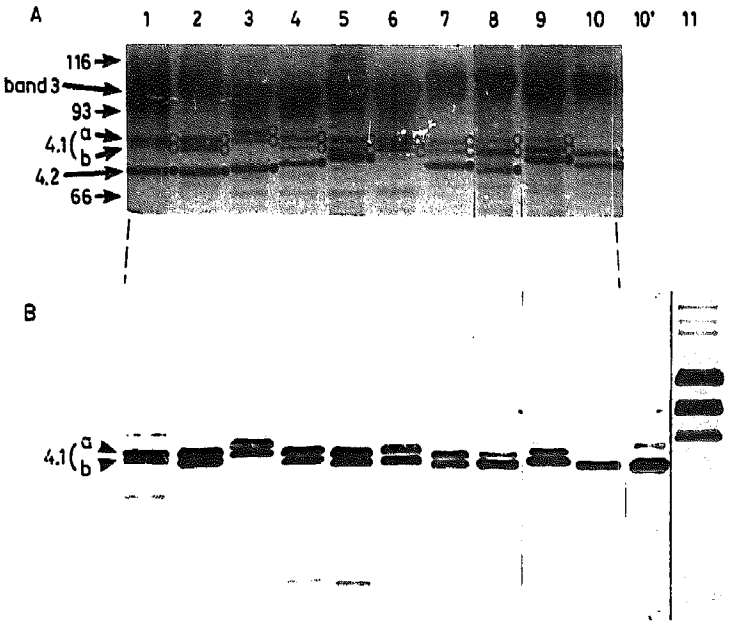

Fig. 1. Identification of protein 4.1 on SDS-PAGE (A) and immunoblotting (B). Membrane proteins from various mammals were subjected to SDS-PAGE followed by staining with Coomassie blue (A) or by immunoblotting using anti-protein 4.1 (B). Each lane contains 12 μg (A), 2 μg (B, lanes 1–10 and 11), or 4 μg (A), 2 μg (B, lanes 1–10 and 11), or 4 μg (B, lane 10′) of proteins. Lanes 1–11 contain membranes from human, dog, sheep, goat, cow, horse, pig, mouse, rabbit, cat and chicken, respectively. Protein 4.1 (○) and 4.2 (●) are indicated. Molecular weights of standards (Bio-Rad) are shown $\times 10^{-3}$.

less than that in avian erythroid protein 4.1; chicken erythroid cell contains multiple variants of protein 4.1 (Fig. 1, lane 11 (reported originally in Ref. 19)) governed by alternative splicing [20].

Relationship between protein 4.1a/4.1b ratio and erythrocyte survival

Densitometric scanning showed that the 4.1a/4.1b ratio was constant in each species, but different in the various mammalian species, as shown in Table I. The values of the 4.1a/4.1b ratios in these mammalian erythrocytes were plotted against the mean erythrocyte survival (Fig. 2). Data on mean erythrocyte survival employed here are shown in Table II. These are the values for adult subjects obtained by labeling erythrocytes with several isotopes [21–40], except for the values for pig erythrocytes which were determined for growing swine [41,42]. Although it is unfortunate that no real life span could be determined in the animals used in the present study, we may apply these reported survival values to our case because, as shown in Table II, the erythrocyte of each species has a characteristic mean survival time in spite of the differences in the procedures used to measure life span. In addition, the animals we used are all very popular domestic or experimental animals.

Mammalian species having a mean erythrocyte life span of more than 100 days (cow, horse, human, goat, sheep and dog) showed higher values for the 4.1a/4.1b ratio, ranging from 1.2 to 1.8, while a 4.1a/4.1b ratio of less than 0.5 was observed in mouse and rabbit, which also had shorter erythrocyte survivals. Pig erythrocytes showed characteristics intermediate between the two groups described above. These results demonstrate that the protein 4.1a/4.1b ratio is correlated to the life span of the erythrocytes, indicating that alteration of the 4.1a/4.1b ratio reflects changes in the population of young or aged erythrocytes in each mammalian species. As shown in the inset of Fig. 2, for the correlation between the ratio of 4.1a/4.1b and the life span of erythrocytes from

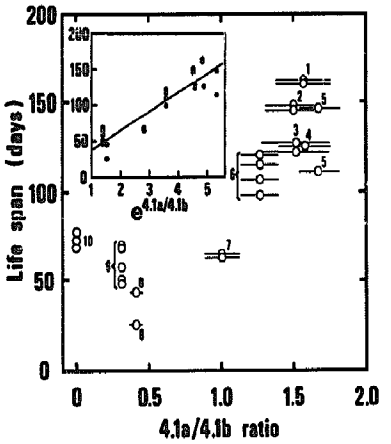

Fig. 2. Correlation of the 4.1a/4.1b ratio and erythrocyte life span. The 4.1a/4.1b ratio (mean ± S.D.) is plotted against the erythrocyte mean survival of each animal. The numbers 1–10 indicate cow, horse, human, goat, sheep, dog, pig, mouse, rabbit and cat, respectively. In the inset, the values of $e^{4.1a/4.1b}$ are plotted against the mean life span values. The different survival values for each animal species, shown in Table II, were employed.

mammals except cat, we could tentatively adapt the following regression formula:

Life span (days) = 26.3 $e^{4.1a/4.1b}$ + 10.4 (r = 0.91)

Proteins 4.1a and 4.1b and their variants in young or precursor cells in erythroidal development

Fig. 3 shows the immunoblots of protein 4.1 from canine reticulocyte membranes and a membrane preparation of K562 erythroid progenitor cells derived from human chronic myelogeneous leukemia [43–45]. In the reticulocytes, protein 4.1b was predominant and faint bands of 4.1a were observed on immunoblots and Coomassie blue-stained gels. Additional polypeptides (77 kDa and 134 kDa) immunoreacted with anti-4.1. The 134 kDa polypeptide seems to be a major variant of protein 4.1 in canine lens cells (data not shown) as reported for bovine lens by Aster et al. [46]. Moreover, a haploid set of polypeptides with molecular masses of 84 kDa and 87 kDa were immunospecifically detected as minor variants of protein 4.1. These two polypeptides are designated as protein 4.1a+ (87 kDa) and 4.1b+ (84 kDa). It should be noted that the ratio of protein 4.1a+ /4.1b+ appeared to correspond to the 4.1a/4.1b ratio in each subject.

Protein 4.1 in K562 cells appears to consist mainly of 4.1b (79 kDa) and an additional protein with a lower molecular mass (77 kDa). In human cells, as is also the case for dog cells, 4.1b (85 kDa) and 4.1a+ (87 kDa) were observed as prominent variants of protein 4.1 in K562 cells and erythrocytes, respectively.

TABLE II

MEAN ERYTHROCYTE LIFE SPANS IN VARIOUS MAMMALS

Species	Life span (days) (n)	Isotopes	Ref.
Human	127	[^{15}N]glycine	21
	121.7 ± 8.7 (6) [a]	[^{32}P]DFP [d]	22
Dog	97.6 ± 7.6 (7) [a]	[^{14}C]glycine	23
	115	[^{14}C]lysine	24
	120 ± 1.7 (3) [a]	^{55}Fe and ^{59}Fe	25
	107 ± 1.1 (5) [b]	$^{55}FeCl_2$	26
Sheep	146 ± 13.5 (3) [a]	[^{14}C]glycine	27
	111.1 ± 26.3 (14) [a]	$^{59}FeCl_3$	28
Goat	125	[^{14}C]glycine	29
Cow	160	[^{14}C]glycine	30
	162	[^{14}C]glycine	31
Horse	145 [c]	[^{14}C]glycine	32
	147.2 ± 8.2 (10) [a]	[^{32}P]DFP	33
Pig	62.3 ± 6.3 (5) [a]	[^{14}C]glycine	41
	63 ± 16 (18) [a]	$^{59}FeCl_3$	42
Rabbit	65 –70 (5)	[^{15}N]glycine	34
	50 ± 0.2 (3) [b]	[^{14}C]glycine	35
	57.2 ± 0.2 (3) [b]	$^{59}FeCl_3$	35
	67.6 ± 1.9 (12) [b]	$^{55}FeCl_3$	26
	45–50 (4)	$^{55}FeCl_3$	36
	50	^{55}Fe	37
Mouse	20–30 (3)	$^{55}FeCl_3$	36
	25	^{55}Fe	37
	44.6 ± 8.1 (10) [a]	[^{32}P]DFP	38
Cat	72.6 ± 5.1 (4) [a]	[^{14}C]glycine	40
	68.4 ± 1.5 (5) [b]	$^{55}FeCl_3$	26
	77	[^{15}N]glycine	39

[a] Mean ± S.D. reported or calculated from the data described in the text.
[b] Mean ± S.E.
[c] Mean value of two experiments.
[d] DFP, diisopropylfluorophosphate.

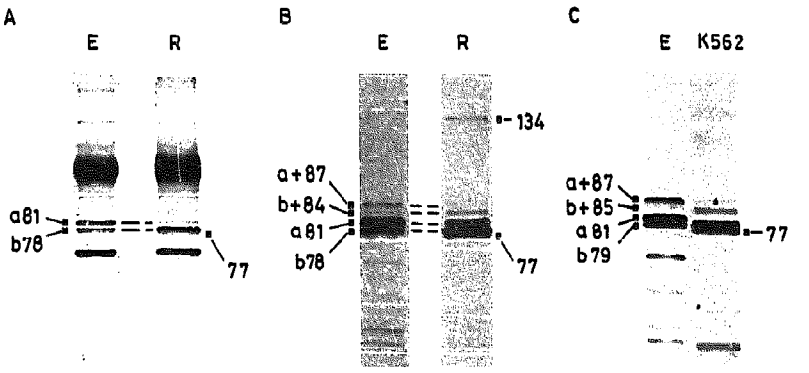

Fig. 3. Protein 4.1 in reticulocytes and K562 cells. Protein 4.1 in dog reticulocytes made anemic by successive bleeding as described previously [14] was analyzed using SDS-PAGE (A) and immunoblotting (B) as in Fig. 1. Reticulocyte purity was 65%. Lanes E and R contain membranes from canine erythrocytes and reticulocytes, respectively. In C, membranes from K562 cells (K; 100 μg) and human erythrocytes (E; 2 μg) were reacted with anti-protein 4.1. Several isoforms of protein 4.1 and their apparent molecular masses are indicated ($\times 10^{-3}$).

Synthesis of protein 4.1 in mouse erythropoietic cells

A single polypeptide (77 kDa) corresponding to protein 4.1 b was observed only when the cytoskeletal fraction from mouse erythroid cells labeled with [^{35}S]methionine was immunoprecipitated with anti-4.1 antibodies (Fig. 4B). Other variant polypeptides, immunorecognized with anti-4.1, i.e., 4.1b + and 4.1a as shown in Fig. 4A, could not be detected in immunoprecipitates from both of the cytoskeletal and the cytoplasmic fractions. Proteins shown in soluble, cytoplasmic fractions were regarded as nonspecifically precipitated materials as they were observed even in the immunoprecipitates with anti-spectrin or anti-ankyrin antibodies (not shown). During the chase period, the amount of protein 4.1b labeled and incorporated into cytoskeleton was gradually increased for 30–60 min and after that no alteration was observed during 180 min.

These results indicate that 4.1b is a major form

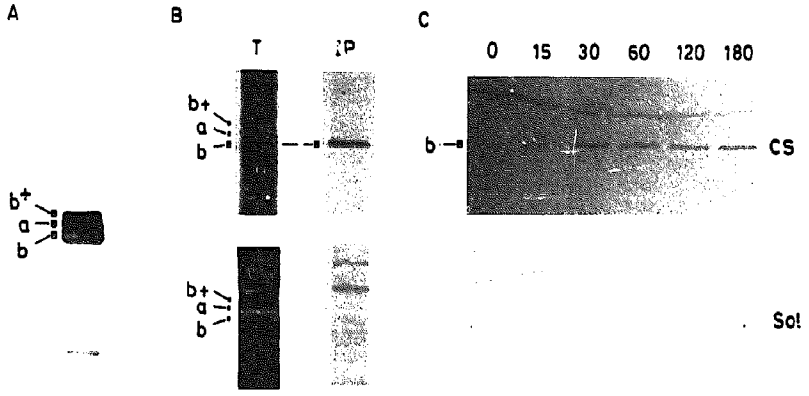

Fig. 4. Biosynthesis of protein 4.1 in mouse erythropoietic spleen cells. (A) Shows the immunoblots of protein 4.1 in mouse erythrocyte membranes (4 μg). Three isoforms of protein 4.1 (b+, a and b) are indicated. Mouse erythroblasts were labeled with [^{35}S]methionine for 60 min (B) or 15 min (C), solubilized and fractionated into cytoskeletal (CS) and soluble, cytoplasmic (Sol) fractions. Labeled proteins were immunoprecipitated with anti-4.1 antibodies and analyzed by SDS-PAGE followed by fluorography. In B, total proteins (T) and immunoprecipitated proteins (IP) are shown. In pulse-chase experiment (C), cells were chased for the indicated time in the presence of 0.5 mM unlabeled methionine after which immunoprecipitation was carried out.

of protein 4.1, synthesized and processed for rapid incorporation into the membrane cytoskeleton in mouse erythropoietic cells.

Discussion

The present study demonstrates that the protein 4.1a/4.1b ratio is closely related to the life span of erythrocytes in most mammalian species. It is known that an increase of the erythrocyte life span results in a population of circulating erythrocytes with an increased mean cell age. Thus, we recognize that the elevated 4.1a/4.1b ratio in erythrocyte membranes from mammalian species with long erythrocyte survival is due to a relative elevation of aged erythrocytes in blood. Until now, changes in the 4.1a/4.1b ratio during red cell aging have been reported in mouse [13] and human [12,47]. Mueller et al. [13] clearly demonstrated, using a serial hypertransfusion technique, that the alteration of protein 4.1 occurs during erythrocyte aging after the interruption of protein synthesis and suggested that protein 4.1a is produced by some sort of posttranslational modification. Our findings of a correlation between the ratio of protein 4.1a/4.1b and the mean erythrocyte survival supports this hypothesis and indicates that the increase of the 4.1a/4.1b ratio depends on the decrease of 4.1b and the simultaneous increase of 4.1a, and this change occurs by a common mechanism in many mammalian species as erythrocytes age. This alteration appears to take place quite slowly. According to the formula proposed in the 'Results,' it takes 81.9 days of mean survival to yield the value of 4.1a/4.1b = 1.0. Thus, if normal distribution reflects cellular age in the peripheral blood of healthy adult mammals, the period required for the equimolar distribution of protein 4.1a and 4.1b might be about 41 days, which is in good agreement with 40 days observed in mouse in vivo red cell aging (Fig. 4 in Ref. 13).

Recently, some investigators have reported the genetic mechanism for the expression of multiple variants of protein 4.1, including 4.1a and 4.1b. In avian erythroid cells, in which protein 4.1 revealed quite a different profile as compared with mammalian protein 4.1 as shown in Fig. 1 and originally in Ref. 19, differential expression of alternative spliced 4.1 gene products have been proposed [20]. Conboy and his co-workers cloned a protein 4.1 cDNA from human reticulocytes [48]. They progressively reported the second cDNA for human erythroid protein 4.1 [49], which contains the extra sequence (an insertion of 102 nucleosides) resulting in the 34 amino acids into the carboxy-terminal domain of the protein, and suggested that the proteins 4.1a and 4.1b may be produced independently from a single gene by a mechanism including alternative splicing of the primary 4.1 RNA transcript. This alternative splicing mechanism is attractive and suitable for the presence of diverse isoforms of erythroid protein 4.1 as shown in Fig. 3. However, their suggestion that 4.1a and 4.1b may be the products of independent mRNAs is incompatible with our results, which indicate that some postsynthetic processing of 4.1b may generate 4.1a as described above.

On the basis of the predominance of protein 4.1b in K562 cells, erythroid progenitor cells [43–45] and in reticulocytes (Fig. 3), protein 4.1 seems to be synthesized as 4.1b at an early stage of erythroidal development. In fact, it was shown that the erythroblasts metabolically labeled with [^{35}S]methionine could produce 4.1b but not 4.1a in both cytoskeletal and cytoplasmic fractions (Fig. 4). These observations appear to favour the two major proteins (4.1a and 4.1b) resulting from posttranslational processing rather than as products of independent mRNAs. Here, it should be noted that the 4.1a+/4.1b+ ratio corresponds well to the 4.1a/4.1b ratio (Fig. 3). In addition, the molecular masses of 4.1a+ and 4.1b+ are similar to those of 4.1a and 4.1b, and these minor isoforms differ by 2–3 kDa as is the case for proteins 4.1a and 4.1b. Based on these results and the demonstration of two different erythroid protein 4.1 cDNAs [49], it can be assumed that proteins 4.1b and 4.1b+ are the products of different mRNAs derived from the single protein 4.1 gene by alternative splicing mechanism, and that protein 4.1a and 4.1a+ are the converted forms of 4.1b and 4.1b+, respectively. The nature of this posttranslational event remains unclear. However, several postsynthetic events which could cause alterations in the apparent molecular mass of proteins on SDS-PAGE have been proposed for protein 4.1 [11,50–54]. Recently, Holt et al. reported that protein 4.1 in human erythrocytes is a unique

intracellular protein containing an *O*-linked GlcNAc moiety in the carboxy-terminal domain [55] to which the difference in 4.1a and 4.1b has been attributed [10]. Furthermore, such a novel structure is common to diverse variants of erythroidal protein 4.1 in several animal species (Inaba, M., Murase, T. and Maede, Y., unpublished results). Further studies on whether some microenvironmental alterations beside *O*-GlcNAc of protein 4.1 could cause the conversion of 4.1b into 4.1a should be required.

We could only detect a single band (77 kDa) as a major component of protein 4.1 in erythrocyte membranes from cats on both SDS-PAGE and immunoblotting (Fig. 1). Results from membranes prepared from fractions obtained by Percoll density gradient separation [56] of feline erythrocytes showed were no different (data not shown). The additive band with a molecular mass of 84 kDa observed on immunoblots (Fig. 1) appears to correspond to 4.1b+ based on its electrophoretic mobility. Hence, we suggest that feline erythrocytes may lack the mechanism for the alteration of protein 4.1 during erythrocyte aging. Thus, we expect that feline erythrocytes could be employed as a model for investigating the molecular basis of this conversion system for protein 4.1, and the functional difference in the two components, 4.1a and 4.1b.

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