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Two novel molecular isoforms of band 4.2 in Japanese Sika deer (*Cervus nippon yesoensis, Heude*) erythrocytes

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Two molecular isoforms of band 4.2 were identified in erythrocyte membranes from 25 Japanese Sika deer (*Ceryus nippon yesoensis, Heude*) based on specific immunorecognition with anti-human band 4.2. These two variants, designated 4.2/78 and 4.2/76, had respective relative molecular weights (M_r) of 78000 and 76000 on sodium dodecyl sulfate-polyacrylamide electrophoresis gels and showed similar profiles after limited proteolysis, exhibiting identity in primary structure. 25 adult Sika deer could be divided into two groups according to the 4.2/78:4.2/76 ratio, indicating a genetic control in the expression of the molecular isoforms of band 4.2. Both polypeptides were completely retained in cytoskeletal protein-depleted membranes and could be removed by alkaline extraction, suggesting that both proteins contribute to the association of membrane proteins.

Band 4.2 is a major component of the human erythrocyte membrane [1]. It is localized on the interior surface of the membrane and accounts for approx. 5% of the total membrane protein [1]. Just as in human erythrocytes, band 4.2 is a predominant erythrocyte membrane protein in various mammals [2]. In all mammalian cells, it has been generally recognized that band 4.2 consists of a single polypeptide with an M_r ranging from 72000 to 80000 on SDS-polyacrylamide gels [1,2]. No observation on molecular diversities of this protein, such as those well established for band 4.1 [2–5], has been reported as yet. Recently, a case of hemolytic anemia was reported in which the patient had red cells lacking band 4.2 completely, suggesting an important role for this protein in stabilizing the red cell membrane organization [6].

Here we show that Japanese Sika deer erythrocyte membranes contain two molecular isoforms of band 4.2 which are immunochemically recognized with anti-human band 4.2. We show that these two proteins are sequence-related polypeptides and are co-extracted from ghosts by the same procedures used for the purification of human erythrocyte band 4.2 [7,8].

SDS-PAGE analysis was performed on 25 adult (more than 1 year old) Sika deer. Erythrocyte membranes from Sika deer contain the well-known predominant proteins of mammalian erythrocyte membranes as observed in human erythrocytes (Fig. 1A) and some additional polypeptides, including gp155 [9]. It was demonstrated that Sika deer could be divided into two groups according to the profiles of the band 4.2 region on SDS-gels. 20 Sika deer had the electrophoretic pattern shwn in the left lane (type I) of Fig. 1A. The M_r of this band 4.2-like protein was calculated to be 78000. In the samples from five other Sika deer, a polypeptide with an M_r of 76000 has appeared in conjunction with a quantitative decrease in the M_r = 78000 protein (type II). These two polypeptides in Sika deer erythrocyte membranes were immunospecifically recognized with anti-human erythrocyte band 4.2 (Fig. 1B). This indicates that the polypeptides with M_r 78000 and 76000 in Sika deer erythrocytes and human band 4.2 might share the antigenic determinant(s). One-dimensional peptide mapping of the two polypeptides showed similar digestion profiles, exhibiting identity in primary structure (Fig. 1C). Based on these results, we determined that the Sika deer erythrocyte membrane proteins with M_r 78000 and 76000 are the sequence-related isoforms of band 4.2. A corresponding polypeptide (M_r 76000) with a small amount in type I membranes appears to be identical to the band 4.2 variant. In the present study, we designated these two isoforms of band 4.2 as band 4.2/78 (M_r = 78000) and band 4.2/76 (M_r = 76000). We can reject the possibilities of proteolytic alteration or artificial conversion of

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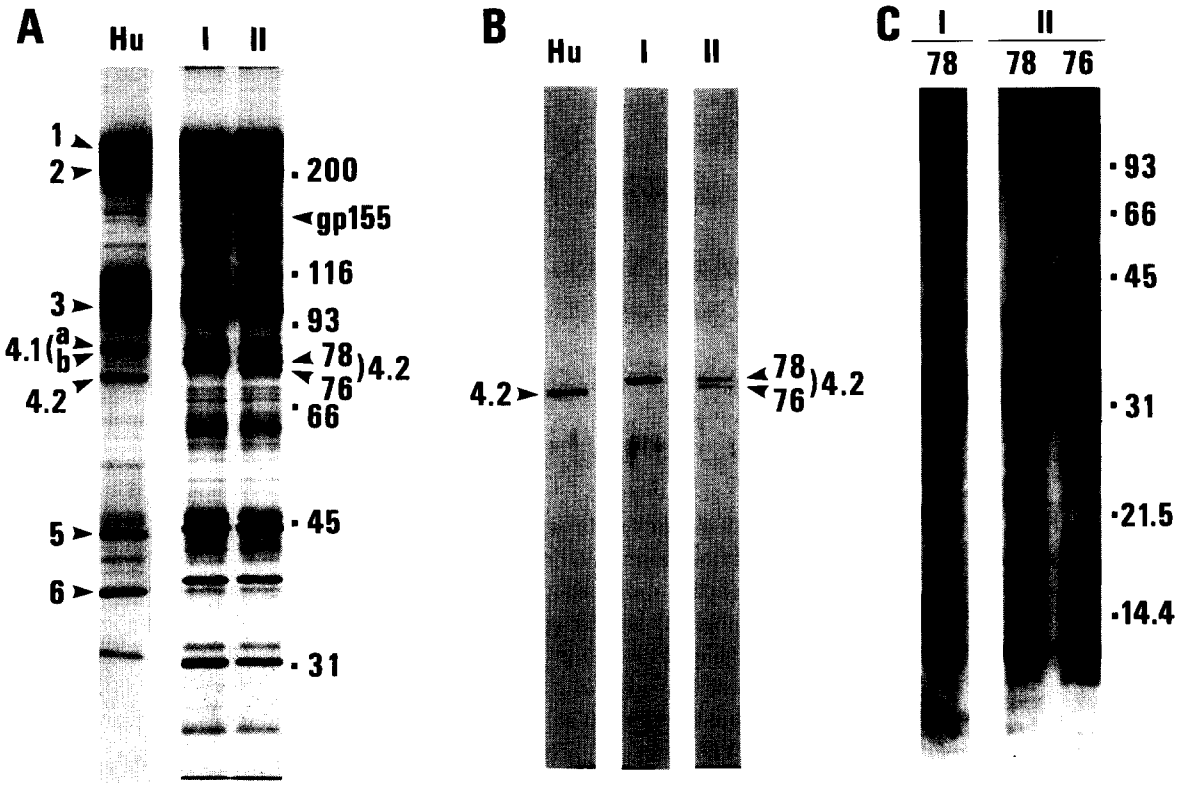

Fig. 1. Identification of two isoforms of band 4.2 in Sika deer erythrocyte membranes. Blood samples were collected under anesthetized condition from 25 (9 males and 16 females) wild adult Japanese Sika deer (*Cervus nippon yesounsis, Heude*) inhabiting Nakanoshima Island of Lake Toya, Japan. Erythrocyte membranes were prepared as described in our previous studies [2,9]. (A) Membrane proteins (25 μg/lane) were analyzed by SDS-PAGE [11] on 8% polyacrylamide gels followed by staining with Coomassie brilliant blue. Lanes I and II show the typical electrophoretic patterns of type I- and type II-membrane proteins (see text). The membrane protein composition of human erythrocytes is also shown (Hu, 20 μg) and the major proteins are indicated (bands 1–6). (B) Band 4.2 were detected by immunoblotting [2,9] using rabbit anti-band 4.2 antiserum. The antiserum was obtained by immunizing a female New Zealand white rabbit with band 4.2 purified from human erythrocyte membranes by the method of Korsgren and Cohen [7] followed by a preparative SDS-PAGE. Lane Hu contains 0.5 μg, lanes I and II contain 10 μg of membrane proteins, respectively. (C) The gel slices containing band 4.2 variants with M_r = 78000 (78) or M_r = 76000 (76) were excised from the initial SDS gel. Limited proteolysis using *Staphylococcus aureus* V8 proteinase (Sigma) and electrophoresis were performed according to the method of Cleveland [12]. After electrophoresis, the gel was stained with silver using a kit from Bio-Rad. Molecular weights of standards (Bio-Rad) are shown $\times 10^{-3}$.

the polypeptides during the membrane preparations because all samples from the 25 deer weer processed under the same conditions, with care taken to minimize the influence of endogeneous proteinases. Thus, the appearance of two band 4.2 isoforms in SDS-PAGE could be assumed to reflect the native state in Sika deer erythrocytes.

Quantitative densitometric scanning of SDS-gels showed that the total amounts of bands 4.2/78 and 4.2/76 account for 3–4% of the total membrane protein mass, and no significant difference of these values was observed between type I and type II Sika deer (Table I). Moreover, the 4.2/78 : 4.2/76 ratio in individual deer was relatively constant in both Sika deer types, as shown in Table I. The type-II group included two males and three females. There were no clinical or hematological disorders such as hemolytic anemia or reticulocytosis. These observations and the recessive frequency of type II (type I/type II = 4 : 1 in the present work) indicate that a genetical control with an autosomal recessive mode might determine the expression of the isoforms of band 4.2. Thus, the mechanism of the expression of band 4.2 variants appeared to differ from that of band 4.1. In the case of band 4.1, an alternative splicing mechanism [10] and a following posttrans-

TABLE I

Relative amounts of band 4.2 isoforms to the total membrane protein mass

Relative amounts of band 4.2 isoforms (4.2/78 and 4.2/76) were estimated by densitometric scanning of SDS gels. Data are expressed as means ± S.D.

Subjects	Band 4.2		
	4.2/78	4.2/76	4.2/78 + 4.2/76
Type I (n = 20)	2.91 ± 0.32	0.42 ± 0.13	3.33 ± 0.39
Type II (n = 5)	1.67 ± 0.25 [a]	1.95 ± 0.28 [a]	3.63 ± 0.50 [b]

[a] Significantly differnet from the value of type I ($P < 0.001$).
[b] No significant difference is observed when compared with the value of type I.

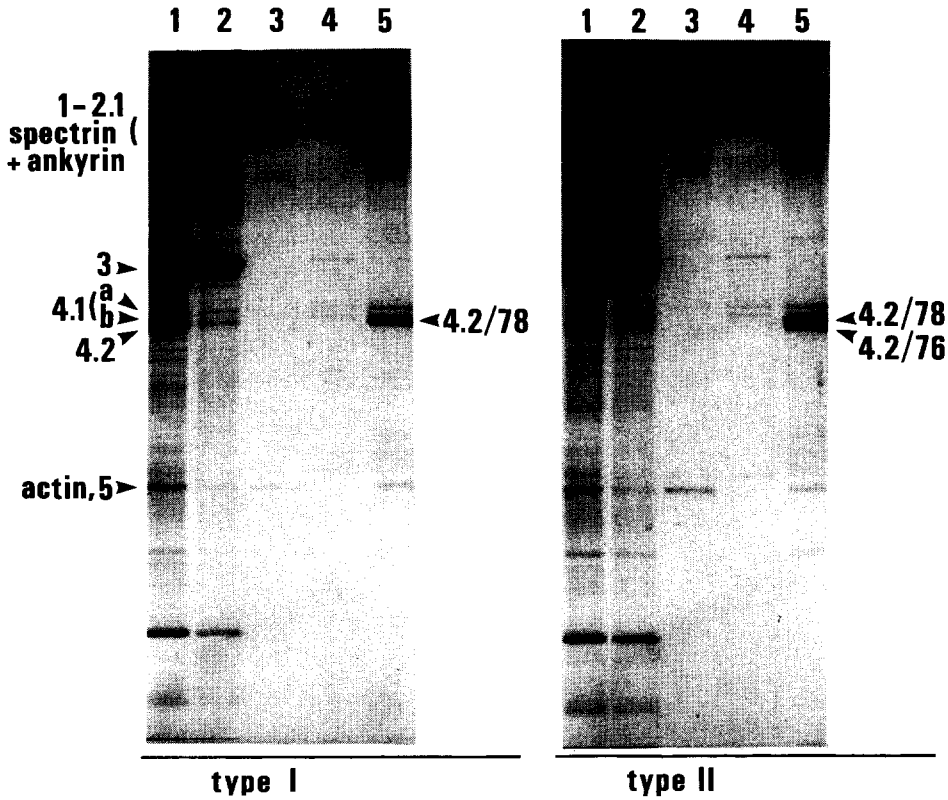

Fig. 2. Retention in peripheral protein-depleted ghosts and extraction into an alkaline buffer of bands 4.2/78 and 4.2/76. Peripheral membrane proteins were extracted from ghost membranes according to the procedures described by previous workers [13,14]. In brief, band 6 was first removed by incubating the membranes in 10 mM sodium phosphate, 154 mM NaCl, 1 mM EDTA, 0.2 mM PMSF (pH 7.4) for 30 min on ice. Ghosts were washed once with 5 mM Tris-Cl/1 mM EDTA/0.8 mM PMSF (pH 7.8). Spectrin and actin were removed by the incubation of band 6-depleted ghosts in 0.1 mM EDTA-Tris/0.8 mM PMSF (pH 8.5) for 30 min at 37°C. After subsequent washing with the same buffer, membranes were treated with 1 M KCl/1 mM EDTA/0.8 mM PMSF/10 mM sodium phosphate (pH 7.6) for 30 min at 37°C. Pelleted membranes were suspended in 0.1 mM EDTA-Tris (pH 8.5), and the pH was adjusted to 11.0 by the addition of 1 M NaOH followed by incubation for 45 min at 37°C [7]. Band 4.2 was extracted into the supernatant after centrifugation at 118000 × *g* for 40 min at 2°C. Samples from each extraction step were analyzed by SDS-PAGE. Lane 1, membrane pellet after 1 M KCl treatment; lane 2, membranes extracted with a pH 11.0 buffer; lane 3, extracts of a low ionic strength (0.1 mM EDTA-Tris) buffer; lane 4, supernatant of 1 M KCl extraction; lane 5, proteins extracted with pH 11.0 buffer in which almost all of both 4.2/78 and 4.2/76 are included.

lational modification [2,5] are responsible for diverse isoforms of erythroid band 4.1. Further studies to clarify the genetic mechanism in band 4.2 are required.

Recently, Korsgren and Cohen [7] purified human erythrocyte band 4.2 using a pH 11.0 medium. They reported that purified band 4.2 binds saturably to the cytoplasmic domain of band 3, to ankyrin, and to band 4.1 and suggested that band 4.2 is implicated in stabilizing the membrane organization [8]. Fig. 2 shows that both bands 4.2/78 and 4.2/76 were completely retained in the inside-out ghosts depleted of cytoskeletal proteins. Almost all of both polypeptides could be removed from the membranes in an alkaline buffer with residual spectrin, ankyrin and band 4.1, as reported for human band 4.2 [7]. This may indicate that Sika deer band 4.2, including both 4.2/78 and 4.2/76, is tightly linked to the membrane. Based on these observations, although there is no direct evidence for the interaction of bands 4.2/78 and 4.2/76 with other membrane proteins, we suspect that two isoforms of band 4.2, 4.2/78 and 4.2/76, may play similar roles in the association of membrane proteins, helping to maintain the cellular integrity and elasticity.

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