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"Protein 4.2: Normal and Abnormal in Clinical Hematology, Protein Chemistry, Molecular Genetics, and Ultrastructure"

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Red cell protein 4.2 (P4.2) is a major component of the red cell membrane skeletal network,[1-6] which binds to the cytoplasmic domain of anion exchanger band 3[7] and interacts with ankyrin in red cells.[8] Patients with P4.2 deficiency in their red cell membranes suffer from congenital hemolytic anemia with microspherocytosis or the like. This fact suggests that P4.2 plays an important role in maintaining the stability and flexibility of red cells. P4.2 was previously reviewed by two authors in 1993[4] and 1994.[5,6] Since then, new information on its genotype and phenotype has extensively accumulated. Therefore, a review of findings on P4.2 and its gene published since 1993 is needed.

Organization of the protein 4.2 gene (ELB42) in human red cells

1. Characteristics of genomic DNA:

The human red cell protein 4.2 gene is ~20 kb and contains 13 exons and 12 introns.[9-12] The coding sequence from the genomic DNA is identical to the cDNA sequence. Nucleotide polymorphism has been observed in the normal human protein 4.2 gene, as shown in Table 1. The exons range in size from 104 to 314 base pairs with an average size of 170 base pairs, while the introns vary from 6.4 to 0.3 kb. All the exon-intron boundaries follow the consensus 5'donor-3' acceptor splice junction sequence for eukaryotic genes of gt-ag. The gene is localized to human chromosome 15q15-q21.[12,13]

The upstream region of the protein 4.2 gene contains several elements[11,12] that are similar in sequence to the upstream elements of the genes for β-globin and porphobilinogen deaminase, which are also red cell protein genes. The elements are spaced a similar distance from the transcription start site and have similar relative spacing and order. These similarities have made it easier to identify five possible regulatory cis-elements in the protein 4.2 gene starting -20 nucleotides upstream from the transcription start site; i.e., (1) a possible TATA element, (2) a short G+C-rich domain, which could be an Sp1 binding site, (3) a possible CAAT box, (4) a CAAC box, and (5) two GF-1 binding domains, one at -23 to -28, and another one at -173 to -178.[11] These findings suggest the use of common cis-elements in these three erythroid genes, although the

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TABLE 1. Nucleotide Polymorphism Observed on the Normal Human Protein 4.2 Gene

	Number of codons														
	-13	140	178	365	366	367	368	369	370	379	380	405	406	Race	References
Sung *et al*	TTT	GCA	GTG	AAG	CGC	GGC	CTG	CCT	TGC	CTG	CAG	ACG	CTG	—	11, 13
		Ala	Val	Lys	Arg	Gly	Leu	Pro	Cys	Leu	His	Thr	Leu		
Korsgren *et al*	CTT	GCG	GTT	ACG	CGG	CCT	GCC	TTG	CCC	CTC	GAC	ACC	GTG	—	10, 12
		Ala	Val	Thr	Arg	Pro	Leu	Leu	Pro	Leu	Asp	Thr	Val		
Takaoka *et al*		GCG	___	ACG	CGG	CCT	GCC	TTG	CCC	___	CAG	___	GTG	J	34
		Ala		Thr	Arg	Pro	Ala	Leu	Pro		His		Val		
Yawata *et al*	TTT	GCG	GTG	ACG	CGG	CCT	GCC	TTG	CCC	CTG	CAC	ACG	CTG	J	U.O.
		Ala	Val	Thr	Arg	Pro	Ala	Leu	Pro	Leu	His	Thr	Leu		

J: Japanese, U.O.: unpublished observation

identification of these elements as having a regulatory function in protein 4.2 gene expression is highly speculative. It has also been reported[12] that the nucleotides upstream from the cDNA start site (nt 1) are (1) CAGT (nt -4 to -1), agreeing well with the CA cap signal, (2) nucleotides -26 to -21 upstream from the cDNA start site having a sequence of ATAAAA, which agrees well in sequence and position with the promoter TATA box for eukaryotic genes, (3) a CCAT sequence was noted at nt -89 to -86 within the reported -385 nt upstream sequence, where upstream promoter elements are located, (4) a GC-rich region from nt -85 to -34 (G/C to A/T ratio=3), (5) within this region, a sequence of CCCACCCC CTCCCCC containing a CACC element (nt -83 to -80) that is a potential binding site for Sp1 nuclear factor, and (6) two AGATAA sequences for potential binding of erythroid-specific transcription factor GATA-1 (also known as GF-1, NF-E1, Eryf-1) located at nt -175 to -170 and at nt -28 to -23, respectively. The number of the 5'-CpG-3' dinucleotide sites appears, unlike the β-spectrin gene,[14] which has numerous 5'-CpG-3' sites as so-called "CpG islands", to be small. The 5'-CpG -3' sites of the protein 4.2 gene were highly methylated, when genomic DNA was prepared from mononuclear cells in normal human peripheral blood.[21]

Alignment of the protein 4.2 amino acid sequence with that of a subunit of human coagulation factor XIII and division of the sequence into exons have revealed a remarkable correspondence,[9-11] although the gene for the a subunit of human factor XIII, which is on chromosome 6p24-p25, is 160 kb and has 15 exons and 14 introns, while the gene for protein 4.2 is only 20 kb and contains 13 exons and 12 introns. With only one exception, the exons of protein 4.2 are very similar and in many cases identical in size to the exons of the a subunit of factor XIII with which they are paired. In addition, in every case, the corresponding intervening introns are of the same splice junction class. These and other similarities suggest that the gene for protein 4.2 is closely related to and possibly derived from that for the a subunit of factor XIII and that the proteins may share common structural and functional properties. However, it should be noted that, despite this close similarity, purified protein 4.2 has no transglutaminase activity in vitro,[9,10] and that normal red cell membranes do not contain transglutaminase activity.[9,10] The lack of protein

4.2 transglutaminase activity is induced by the substitution of the cysteine (GQCWVF) at the highly conserved consensus sequence in the transglutaminase, which is substituted for an alanine (GQAWVL) in protein 4.2. The cysteine appears to be required for transglutaminase activity.[9,10] It is also possible that the substitution of a leucine for a phenylalanine may also be responsible for a loss of this activity.

It has been shown that reticulocytes contain two forms of protein 4.2 mRNA, a small form (P4.2S) encoding a protein of 691 amino acids, and a larger form (P4.2L), which contains an additional 90 nucleotides following nucleotide +9, encoding a protein of 721 amino acids.[11,12] Protein 4.2 exon I contains a 5' noncoding sequence, the translation start site, and 99 nucleotides encoding 33 amino acids. These 33 amino acids are identical to the first 33 amino acids of the larger protein 4.2 transcript. The 3'-most 90 nucleotides of exon I, coding for 30 amino acids, are removed by splicing to generate the smaller transcript, coding for the 691 amino acid protein (a wild type of protein 4.2 : 72 kD on the SDS-PAGE).

The genomic organization of the protein 4.2 gene of human red cells contains 13 exons ; i.e., exon I : ut ~33 residues, II : 34~95, III : 96~173, IV : 174~213, V : 214~248, VI : 249~307, VII : 308~354, VIII : 355~388, IX : 389 ~469, X : 470~569, XI : 570~623, XII : 624~668, and XIII : 669~ut (ut : untranslated sequence).[9-11] The sizes of the introns were 6490 for intron 1, 900 for 2, 580 for 3, 340 for 4, 940 for 5, 320 for 6, 740 for 7, 500 for 8, 390 for 9, 2560 for 10, 2200 for 11, and 3080 for 12.[11]

2. cDNA of the protein 4.2 gene :

Protein 4.2 complimentary DNA (cDNA) obtained from a human reticulocyte cDNA library has been cloned and sequenced.[9,10,12]. The full-length cDNA was 2.35 kb and contained an open reading frame with a 227 - nt untranslated region upstream from the putative ATG start codon. The calculated molecular weight was 76.9 kD encoding 691 amino acids. The nucleotide sequence CAACCATGC around this initiation site was similar to the consensus sequence for initiation found in higher eukaryocytes, except that the second nt in the P4.2 cDNAs was A rather than C.[10] The presence or absence of the 90 nt insert gave rise to two P4.2 cDNA sequences (P4.2S) from 2073 bp and P4.2L from 2348 bp.[11,12]

The amino acid sequence derived from the 2.5 kb cDNA contained ~43% nonpolar, ~35% polar, ~10% acidic, and ~12% basic amino acid residues.[10] The most abundant amino acids were leucine (82 residues) and alanine (60 residues). There were 49 serine and 43 threonine residues, which are potential sites for O-glycosylation and represent 13% of the total residues.[10] There were 16 cysteine residues, 6 potential N-glycosylation sites (Asn-Xaa-Ser/Thr) at Asn -103, -420, -447, -529, -604, and -705, one potential cAMP-dependent phosphorylation site (basic-basic-Xaa-Ser) at Ser-278, and 9 potential protein kinase C phosphorylation sites (Ser/Thr-Xaa-Arg/Lys) at Ser-7, -57, -58, -154, -224, -449, -455, and -666, and Thr-287.[10] There was one Arg-Gly-Asp sequence at 518-520.

Secondary structure analysis predicted that P4.2 should contain ~33% β-sheet, ~24% α-helix, and ~45% reverse turns. Hydropathy analysis of the

deduced amino acid sequence revealed a major hydrophobic domain (residues 298-322), which was predicted to be mainly a β-sheet structure with a possible turn. There was a strongly hydrophilic region (residues 438-495). Toward the C terminus of this region, there was a highly charged segment predicted to be an α-helix (residues 470-492) and containing a large number of both positively and negatively charged residues, especially glutamic acid.[10] Elsewhere, it was reported that there were 37% hydrophobic residues and 28% polar residues.[9] Protein 4.2 did not show any obvious repeating primary structure, but a globular protein was suggested.[9] There were no extended stretches of β-sheet or α-helix. Instead, the protein was characterized by short segments. A hydropathy plot of protein 4.2 showed short alternating regions of hydrophobic and hydrophilic character.[9] The region of the protein between amino acids 265 and 475, however, was characterized by two sets of alternating, prominent hydrophobic and hydrophilic domains.[9]

3. The protein 4.2 gene in mouse red cells

Among the three reports published on the protein 4.2 gene in mouse red cells, there have been substantial discrepancies.[22-24] Korsgren and Cohen (1994) described isolation of a 3.5 kb mouse P4.2 cDNA with the P4.2 transcript of 4.1 kb from mouse reticulocytes.[22] Rybicki *et al* (1994), on the other hand, reported isolation of a full-length P4.2 cDNA of 2.2 kb from mouse reticulocytes.[23] Karacay *et al* (1995) recently described an entire P4.2 cDNA sequence consisting of 3465 nt with an open reading frame (ORF) of 691 amino acids, and despite its similarity to human P4.2 cDNA, the mouse cDNA had a longer 3' untranslated region.[24] In addition, they reported that the mouse reticulocyte P4.2 RNA did not exhibit alternative splicing in the region identified in human P4.2 RNA.[24] The P4.2 gene in mice was mapped to murine chromosome 2,[25] in contrast to 15ql5-q21 in the human red cell P4.2 gene.[12,13]

4. Tissue-specific expression of the mouse protein 4.2 gene and the pallid mutation :

Immunoreactive forms of P4.2 with a molecular weight of 72 kD, or larger or smaller than 72 kD, have been detected in nonerythroid cells and tissues.[4,26-28] Immunologic cross-reactivity between the red cell P4.2 protein and other cellular proteins has also been reported.[4,26-28]

Zhu *et al* (1998) recently reported that expression of the mouse P4.2 gene was temporally regulated during embryogenesis and that the P4.2 mRNA expression pattern matched the timing of erythropoietic activity in hematopoietic organs.[27] It should be noted that, contrary to previous reports,[4,22,28] P4.2 expression was detected only in the erythroid cell-producing organs and circulating red cells during mouse embryonic development and in adult mice.[27] They first analyzed poly A^+RNAs from various adult mouse tissues by Northern blot analysis using a 714 bp mouse P4.2 cDNA fragment containing the 3' protein of the P4.2 -coding region as the probe. A single 3.5 kb P4.2 transcript was detected at relatively high level in the spleen, while little or no P4.2 hybridization was seen in other tissues examined (brain, lung, liver, skeletal muscle, kidney, or testis). They extended the use of mouse embryos for their P4.2 expression studies. A P4.2 hybridization signal was first detected not

at E6.5 days, but instead in primitive erythroid cells in E7.5 embryos. In E10.5 embryos, the P4.2 hybridization signal was detected only in the heart and blood vessels. In E12.5 embryos, there was a switch in the hematopoietic production sites from the yolk sac to the fetal liver. In E 16.5 embryos, the signal was greatly reduced in the liver, and was almost undetectable after birth. Finally, P4.2 gene expression became confined to the red pulp on postnatal day 7. No P4.2 specific labeling was observed in the white pulp, which consisted of germinal centers for lymphocytes, plasma cells, and macrophages. No P4.2 hybridization signal was detected in megakaryocytes. Therefore, the P4.2 message was specifically expressed in cells of erythroid lineage in postnatal hematopoietic organs.[27]

The chromosomal location of the mouse P4.2 gene was near a mouse pallid (pa) mutation.[28] Pallid was found in a mouse with dilution of coat color, increased bleeding time, and abnormal lysosomal enzyme secretion, as a model of the platelet storage pool disease.[29,30] Therefore, it has been suggested that the P4.2 gene may be related to the pallid mutation gene,[4,22,28] and it has even been proposed that the P4.2 gene itself should be nominated as a "pallidin".[4,22,28] Patients with P4.2 deficiency,[5,6,31–39] however, do not have the platelet storage pool deficiency seen in pa/pa mice, and the mutant mice do not exhibit the hemolysis and spherocytosis observed in the P4.2 deficiency. It has recently been shown that the P4.2 gene is distinct from the pa gene,[25] and that changes in P4.2 in pallid mice were not responsible for the pallid mutation.[25,27] There have also been reports of P4.2 transcripts, in addition to spleen, in other tissues (kidney, heart, brain, and liver), and even in other cells (HeLa cells or HT-29 cells).[4,26–28] Like the results with the immunoreactive forms of P4.2 previously detected in nonerythroid tissues or cells,[4,26–28] neither P4.2 message nor protein 4.2 have been found in nonerythroid tissues and cells.[27]

Protein chemistry of normal red cell membrane protein 4.2

Protein 4.2 is a membrane protein accounting for approximately 5% of total membrane protein content and for 250×10^3 copies per red cell.[40] It has a molecular weight of 72 kDa on SDS-PAGE.[41–43] Excellent reviews have been published on normal protein 4.2[4,5] and on some disease states of protein 4.2.[5,6]

Extraction of protein 4.2 from red cell membranes is more difficult than for any other peripheral protein, even under high and low ionic strength conditions. Therefore, strong basic conditions (pH 11 or above) have been used in combination with gel filtration with 1 M KI-Sephacryl S-200 as the standard method for the extraction of protein 4.2.[7] This procedure yields 1-2 mg of 'type I' protein 4.2 from 500 ml of whole blood with a purity of approximately 85%. This protein is water-soluble, and difficult to separate from residual ankyrin and protein 4.1. The use of other extraction methods with 10 to 20 mM lithium diiodosalicylate, 6 mM 2,3-dimethylmaleic anhydride, 5 mM *p*-chloromercuribenzoic acid (pCMB), or 1 mM *p*-chloromercuribenzoic sulfate (pCMBS) have been reported.[4,44–46] The non-ionic detergent Triton X-100 can also be used to extract protein 4.2 from red cell membranes.[47,48] Under this condition, band 3 is co-extracted with the portion 4.2 portion, suggesting an

association of protein 4.2 with band 3 in red cell membranes in situ.

An alternative method using 2 M Tris-HCl (pH 7.6) has recently been used to extract protein 4.2 with a purity of more than 97%. However, this 'type II' variety of protein 4.2 is less water-soluble and behaves like an integral protein.[49] It is speculated that its characteristic hydrophobicity is due to myristylation.[4]

Protein 4.2 purified by the standard pH 11 method with 1 M KI-Sephacryl S-200[7] appears heterogeneous in size and is probably primarily comprised of a mixture of dimers and trimers.[4] Electron microscopically, purified protein 4.2 appears as globular particles with diameters in the range of 80-150Å,[4] and has been suggested to be tetrameric in situ in the membrane.[42,50]

Protein 4.2 in human red cell membranes is known to be myristylated at a site near the N-terminus,[4,51] as assayed by the release of myristoyl glycine from partially hydrolyzed protein 4.2.[52] Glycine at the second position appears to be responsible for this myristylation (*N*-myristoyl glycine).[53] Further studies of its biological functions should be considered.

It has also been reported that protein 4.2 is palmitoylated in a physiological condition.[54] After labeling of intact human red cells with [^{3}H] palmitic acid, radioactivity was found to be associated with protein 4.2 by immunoprecipitation of peripheral membrane proteins extracted at pH 11 from ghosts with anti-protein 4.2 antibody. The fatty acid linked to protein 4.2 was identified as palmitic acid. Protein 4.2 could be depalmitoylated with hydroxylamine, suggesting a thioester linkage. Depalmitoylated protein 4.2 showed significantly decreased binding to protein 4.2-depleted membranes, as compared with native protein 4.2. Several red cell membrane proteins including ankyrin, band 3, p55, protein 4.1 and spectrin were palmitoylated. Fatty acid acylation of proteins confers an extra hydrophobic moiety to proteins, which promotes hydrophobic protein-membrane and protein-protein interactions. Whereas control protein 4.2 showed a binding capacity of 280 mg/g vesicle protein (band 3), depalmitoylated protein 4.2 showed capacity of 108 mg/g vesicle protein.[54] Therefore, palmitoylation of protein 4.2 appears to favor its interaction with band 3 in the membrane.

To date, protein 4.2 has not been crystallized. Therefore, crystallography is expected to be performed in the future.

Functions of protein 4.2

The major functions of protein 4.2 are in association with other membrane proteins topographically adjacent to it *in situ* in red cell membranes, especially band 3, ankyrin, spectrin, and protein 4.1.

1. Binding properties of protein 4.2

i) Interactions of protein 4.2 with band 3

When Triton X-100 extracts of red cell membranes were fractionated by ion exchange chromatography and non-denaturing gel electrophoresis, protein 4.2 was found with band 3.[48] In addition, direct binding assays have indicated that an excess cytoplasmic domain of band 3 abolished the normal ($2\text{-}8 \cdot 10^{-7}$ M) binding of purified protein 4.2 to red cell inside-out vesicles (IOV). The binding of protein 4.2 to the purified cytoplasmic domain of band 3 usually

takes 6 to 20 h for complete saturation.[8] Therefore, it has been hypothesized that re-binding of protein 4.2 to the membrane probably requires the formation of other types of associations besides protein-protein contacts at the membrane-medium interface, perhaps the formation of protein 4.2 or band 3 oligomers.[4] Although that the major binding site of protein 4.2 has been considered to be the cytoplasmic domain of band 3, no direct evidence has been found,[4] because the state of self-association of purified protein 4.2 is heterogeneous and the exact oligomeric state of band 3 *in situ* in membranes under the conditions of the binding assays is unknown. It has been tentatively estimated that the stoichiometry of protein 4.2 to band 3 interaction is approximately 1 : 3.9 on a monomer basis.[7]

One synthetic peptide of protein 4.2 (P8 : L^{61} FVRRGQPFTIILYF) was recently found to bind strongly to the cytoplasmic domain of band 3 . Four other peptides (P22 : L^{271} LNKRRGSVPILRQW, P27 : G^{346} EGQRGRIWIFQTST, P41 : L^{556} WRKKLHLTLSANLE, and P48 : I^{661} HRERSYRFRSVWPE) bound less strongly.[55] These peptides have in common a cluster of two or three basic amino acid residues (arginine or lysine) in a region with nearly no acidic residues. The cytoplasmic domain of band 3 bound saturably to P8 with a Kd of 0.16 μM and a capacity of 0.56 mol of the cytoplasmic domain of band 3 monomer/mol of P8. Replacement of R^{64} R with R^{64} G, G^{64} R or G^{64} G almost completely abolished the cytoplasmic domain of band 3 binding, suggesting that R^{64} R is essential for its binding. P8 competitively inhibited binding of purified human erythrocyie P4.2 to the cytoplasmic domain of band 3.

Protein 4.2 can be found with band 3 and ankyrin in an immunoprecipitated complex, probably due to association of protein 4.2 with ankyrin and band 3 *in situ*.[56,57] A partial deficiency of band 3 has also been found to be accompanied by partial deficiency of protein 4.2.[58–61] Furthermore, a cow with total band 3 deficiency[62] and mice with the targeted band 3-knock-out gene[63,64] clearly demonstrated a loss of protein 4.2 in their red cell membranes. The rotational and lateral mobility of band 3 in red cell membranes from patients with protein 4.2 deficiency is substantially increased.[4,5,33,65,66] In our study with fluorescence recovery after use of the photobleaching method (FRAP), the immobile fraction of band 3, which constitutes about 60% of the total in normal red cells, was totally absent in complete protein 4.2 deficiency.[5,33] It is also interesting that, in complete deficiency of protein 4.2, the number of intramembrane particles (IMP) has been found to be reduced with a shift to larger sizes, indicating the possibility of increased oligomerization of band 3 molecules in these red cells.[67] Heat treatment considerably enhanced this effect.[33,67] The structural and functional characteristics of band 3 in these protein 4.2-deficient red cells appeared normal in terms of the cleavage pattern of band 3 fragments and the binding properties of band 3 to protein 4.2 or ankyrin.[5,6,33,67]

ii) Interactions of protein 4.2 with ankyrin

Regarding the association of protein 4.2 with ankyrin, it has been reported that protein 4.2 can bind to 0.65 mol of ankyrin per mol of protein 4.2 with a K_d of 1 to $3.5 \cdot 10^{-7}$ M on the Scatchard plot *in vitro*.[8] However, this binding requires several hours to approach saturation in solution, and no

conclusive evidence has yet been shown for an association of protein 4.2 with ankyrin in the membrane *in situ*.[4] It has also been shown that ankyrin can bind to the cytoplasmic domain of band 3 in IOV without protein 4.2,[4] and that reassociation of ankyrin with IOV is unaffected even when protein 4.2 is removed from the IOV.[4,56]

In some hemolytic anemias, however, a partial deficiency of protein 4.2 has also been reported in ankyrin deficiencies.[68] Decreased protein 4.2 content has been noted in a mouse strain (nb/nb) with an ankyrin deficiency.[69,70] In a protein 4.2 deficiency with the 142 Ala → Thr point mutation, it was reported that ankyrin was partially released from the patient's red cell membranes upon preparation of IOV,[31] suggesting that protein 4.2 might contribute to the stability of the membrane protein association.

It has been shown that the red cell membranes of nb/nb mice, which were nearly completely deficient in full-length 210 kD ankyrin due to a defect in the Ank-1 gene on mouse chromosome 8, were severely (up to 73%) deficient in protein 4.2 content.[69] This deficiency of protein 4.2 in nb/nb homozygous mice was not the result of defective protein 4.2 synthesis. Reconstitution of nb/nb inside-out vesicles with human red cell ankyrin restored ankyrin levels to up to 80% of normal levels and increased binding of exogenously added human red cell protein 4.2 by approximately 60%.[69] These results suggest that ankyrin is required for normal associations of protein 4.2 with the red cell membrane.

iii) Interactions of protein 4.2 with spectrin

Normal protein 4.2 has been shown was found to bind to spectrin in solution and to promote the binding of spectrin to ankyrin-stripped inside-out vesicles. Two independent classes of binding sites of protein 4.2 to spectrin have been identified: 1) a high-affinity ($Kd = 7.4 \pm 0.2 \times 10^{-9} M^{-1}$), low-capacity ($0.6 \pm 0.8 \times 10^{-9}$M/liter) class of sites; and 2) a low-affinity ($Kd = 2.8 \pm 2.0 \times 10^{-7} M^{-1}$), high capacity ($5.8 \pm 1.0 \times 10^{-9}$M/liter) class of sites, as shown in Figure 1. It has been calculated that, at saturation, there is approximately one spectrin binding site per seven protein 4.2 molecules.[65] Therefore, protein 4.2 provides low-affinity binding sites for both band 3 oligomers and spectrin dimers on the human red cell membrane. These observations suggest that protein 4.2 may stabilize skeleton-membrane interactions by providing a direct link between band 3 and spectrin, as shown in Figure 2.

In a disease state, i.e., in red cells with protein 4.2 deficiency,[33,35,36,67] the cytoskeletal proteins (spectrins, ankyrin, and protein 4.1) in red cells of $4.2^{-/-}$ mice[71]) are not deficient. However, the cytoskeletal network in these protein 4.2-deficient red cells appears to be less extended when studied by electron microscopy using the surface replica method and the quick-freeze deep-etching method.[33,67] Interestingly, the cytoskeletal network in these protein 4.2-deficient red cells becomes markedly disorganized, with the appearance of larger aggregates when heat-treated up to 48°C.[5,33,67] Under these conditions, a marked decrease in red cell membrane defomability has been observed by ektacytometry.[5,6,33] It should be noted that, the content of spectrin and ankyrin were maintained normally in these cells.[5,6,33] Therefore, these abnormalities appear to be independent of spectrin and ankyrin per se, and due to the lack of protein 4.2. These results raise the possibility that protein 4.2 may play a

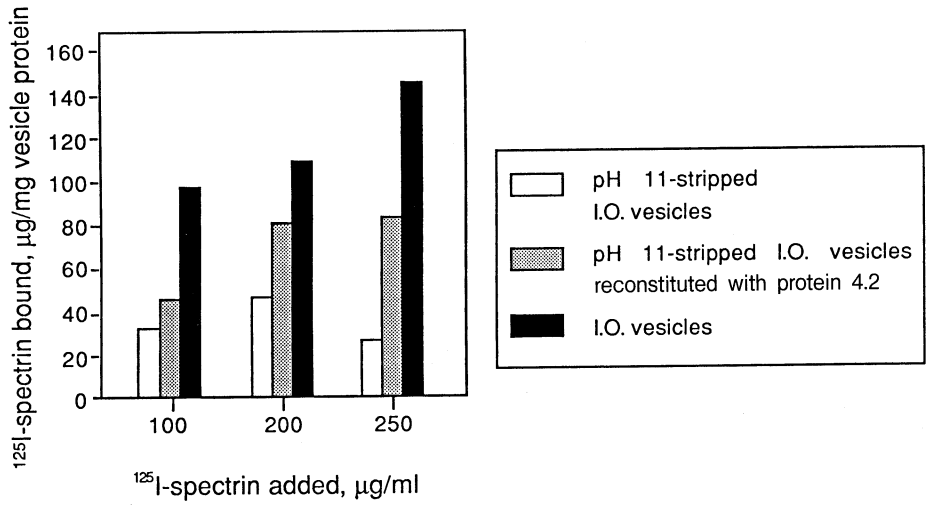

Fig 1. Binding of normal ^{125}I-spectrin to normal protein 4.2 in solution.
Spectrin was iodinated to specific activity of 2840 cpm/μg. The inset shows a Scatchard plot of the data, which were fitted to a two-component model by using non-linear regression analysis. The two binding sites had the following characteristics: Site 1, $Kd=7.4\times10^{-9}$ M^{-1}, capacity$=0.6\times10^{-9}$ M/l; site 2: $Kd=2.8\times10^{-7}$ M^{-1}, capacity$=5.8\times10^{-9}$ M/l. Control binding experiments showed that the antiserum immunoprecipitated 4×10^{-8} M ^{125}I-protein 4.2.[65]

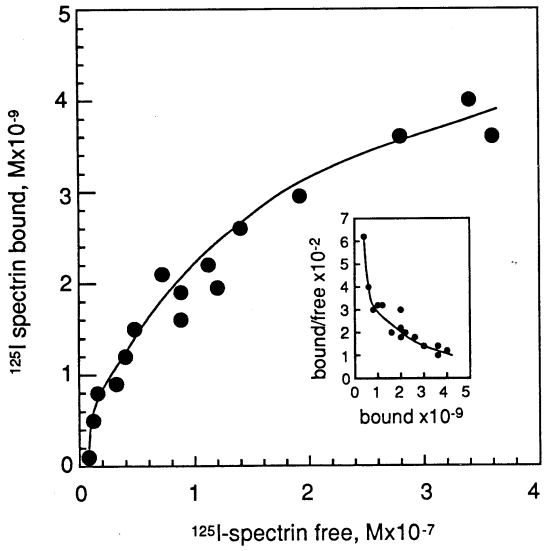

Fig 2. Binding of normal ^{125}I-spectrin to normal protein 4.2 in reconstituted membranes.
pH 11-stripped membranes were prepared and reconstituted with purified protein 4.2. Binding of ^{125}I-spectrin, specific activity 12,096 cpm/μg, to membranes was corrected for non-specific binding. The protein 4.2/band 3 ratios on the membranes, determined by densitometry of Coomassie blue stained SDS gels, were: pH 11-stripped membranes, 0.02; inside-out vesicles, 0.15; protein 4.2 reconstituted inside-out vesicles, 0.45. Data presented in this Figure were representative of those obtained in five independent experiments using different preparations of spectrin, protein 4.2, and membranes.[65]

role in connecting the cytoskeletal network to integral proteins (especially band 3) as a kind of anchoring protein.[5,6,33,65–67]

iv) Interaction of protein 4.2 with protein 4.1

As to a possible association of protein 4.2 with protein 4.1, it has been reported that protein 4.1 may interact with protein 4.2 in solution,[8] suggesting masking of the binding domains of these proteins when they are present together in solution.[4] Protein 4.2, protein 4.1 and ankyrin binding are partially inhibited (about 50%) by the presence of these proteins.[4]

The protein 4.1 and protein 4.2 binding sites have been localized to nearby sites on the cytoplasmic domain of band 3.[72] It is possible that, in the absence of protein 4.2, additional binding sites for protein 4.1 on band 3 may be exposed. The content of protein 4.1, however, remained nearly normal both in human red cells with total deficiency of protein 4.2[5,6,33] and in $4.2^{-/-}$ mouse red cells.[71]

It is also noteworthy that the content of protein 4.2 appeared to be normal in mice with complete deficiency of all protein 4.1 R isoforms, which was generated by gene knock-out technology.[73]

2. Transglutaminase activity of protein 4.2

Protein 4.2 in human red cells has no transglutaminase activity, although the homology in the gene structure between protein 4.2 and transglutaminase[9–11] is high (i.e., an overall identity of 32% in a 446 amino-acid overlap to guinea-pig liver transglutaminase, and of 27% in a 639 amino-acid overlap to human coagulation factor XIII subunit a). It has been speculated that the lack of transglutaminase activity may be due to the presence of an alanine substituted for the cysteine at the active site of the molecule.[9–11]

3. Phosphorylation of protein 4.2

Although phosphorylation has not been observed on protein 4.2 extracted from mature human red cells, protein 4.2 can be phosphorylated, if it is purified by methods that result in exposing phosphorylation sites through alteration of protein 4.2 sulfhydryl groups.[4,49] Seventeen potential phosphorylation sites have been activated in red cell ghosts (i.e., eight possible protein kinase C sites, seven casein kinase II sties, one tyrosine kinase site, and one cAMP-(or cGMP-) dependent kinase site.[49] It has been suggested that membrane-associated protein 4.2 in human mature red cells is already fully phosphorylated with little or no turnover under normal conditions.[49] The physiological functions of such potential protein 4.2 phosphorylation is unknown.

The activities of the major red cell kinases have recently been determined to assess the phosphorylation status in red cells of $4.2^{-/-}$ mice.[71] Cytosolic protein kinase C (PKC) was significantly decreased with decreased PKC-α and PKC-β I isoforms and normal PKC-β III in $4.2^{-/-}$ red cells. Cytosolic protein kinase A (PKA) activity was increased in these red cells. Basal phosphorylation was increased and PMA-stimulated phosphorylation was reduced in $4.2^{-/-}$ red cells. Cytosolic casein kinase I (CKI) activity was normal, but cytosolic CK II activity was decreased in these red cells. The functional significance of these activities remains to be clarified in the future.

Protein 4.2 in red cell membrane ultrastructure

Although the localization of P4.2 in the red cell membrane structure still has not been elucidated in detail, there are several suggestive pieces of evidence. In total deficiency of P4.2, it is now recognized that the intramembrane particles (IMPs) are clustered in the inside-out vesicles (IOVs) of the patient's red cells. In addition, electron microscopic studies with the freeze fracture method[5,33,67] have shown IMPs on red cell ghosts to be enlarged, suggesting increased oligomerization of band 3 molecules. This phenomenon has been verified by biophysical analyses.[5,6,33] Furthermore, analyses with the quick-freeze deep-etching method or the surface replica method have shown the cytoskeletal network to be disrupted in this disorder.[5,33,67] These results appear to indicate that P4.2 molecules are located near band 3 molecules and membrane proteins making up the cytoskeletal network. Biophysical analyses, especially with ektacytometry, have revealed the increased instability of the cytoskeletal network of the patient's red cells.[5,6,33] It has recently been proven that P4.2 can bind directly to spectrins,[65] implying that P4.2 may play an important role as one of the anchoring proteins connecting the cytoskeletal network to the integral proteins (especially band 3 molecules).[5,65–67]

The exact localization of various membrane proteins *in situ* in the normal human red cell membrane ultrastructure has been studied by immnuno-electron microscopy with the surface replica method by utilizing antibodies against various membrane proteins; i.e., spectrins, ankyrin, band 3 (the cytoplasmic domain), protein 4.1 and protein 4.2,[74] as shown in Figure 3. At first, spectrins were easily detected as major constituents of the cytoskeletal network, and ankyrin and protein 4.1 were also identified on it. Band 3 molecules were found attached to the cytoskeletal network as an immobile form of band 3. Other band 3 molecules were located inside the basic units as band 3's mobile form. P4.2, however, could not be detected by this procedure, when the antibody-conjugated immunogold particles were applied to the open red cell ghosts, implying that the epitopes of P4.2 were absent. Therefore, normal red cell ghosts were subjected to gentle treatment with triton X100 to remove a part of the cytoskeletal network. P4.2 was then found to be mostly attached to the cytoskeletal network, as shown in Figure 4. These results suggest that P4.2 is very likely present at the outer face of the cytoskeletal network and even under the lipid bilayer, and is attached to spectrins and band 3, as shown by biophysical studies.[65,66]

Expression of protein 4.2

1. Expression of red cell membrane protein 4.2 and its mRNA in normal human erythroid maturation

The expression of membrane proteins in erythroid cells has been studied in various species: i.e., in avian cells transformed by the avian erythroblastosis virus (AEV) or S-13,[75–80] in murine erythroleukemia cells (MEL),[81,82] and in erythroid progenitors transformed by the Friend virus (FVA)[83–85] or Rauscher erythroleukemia virus,[86] which can differentiate to mature erythroblasts. It has become clear that spectrin as a cytoskeletal protein is already expressed in early erythroid progenitors, followed by sequential expression of band 3 as an

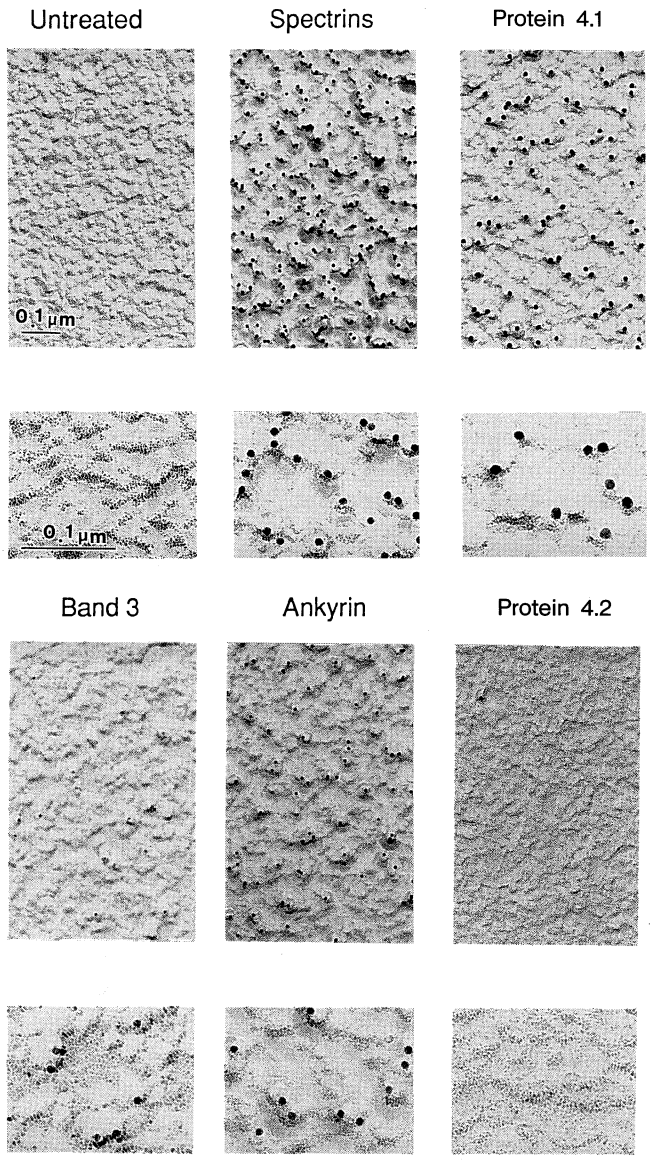

Fig 3. Localization of membrane proteins on the red cell membranes of normal human subjects detected by immunoelectron microscopy using the surface replica method with anti-membrane protein antibodies.

For immunogold labeling of red cell membrane proteins, the following antibodies were used for this study: antihuman spectrin rabbit polyclonal IgG antibody; antihuman protein 4.1 mouse monoclonal IgM antibody, which was provided by Dr. W. Nunomura (Tokyo Women's Medical College, Tokyo, Japan); monoclonal IgG antibody against the cytoplasmic domain of human red cell band 3 (Sigma Chemical Co., St. Louis, MO); antihuman ankyrin rabbit polyclonal IgG antibody, which was provided by Prof. S. Lux (Children's Hospital, Boston, MA); and rabbit polyclonal affinity-purified antibody against human red cell protein 4.2.[67,89,109]

The upper cases denote the pictures of the lower magnification, and the lower cases depict those of the higher magnification in each category.

Protein 4.2, as shown in the cases at the right side end, was not detected even in the presence of anti-protein 4.2 antibody.

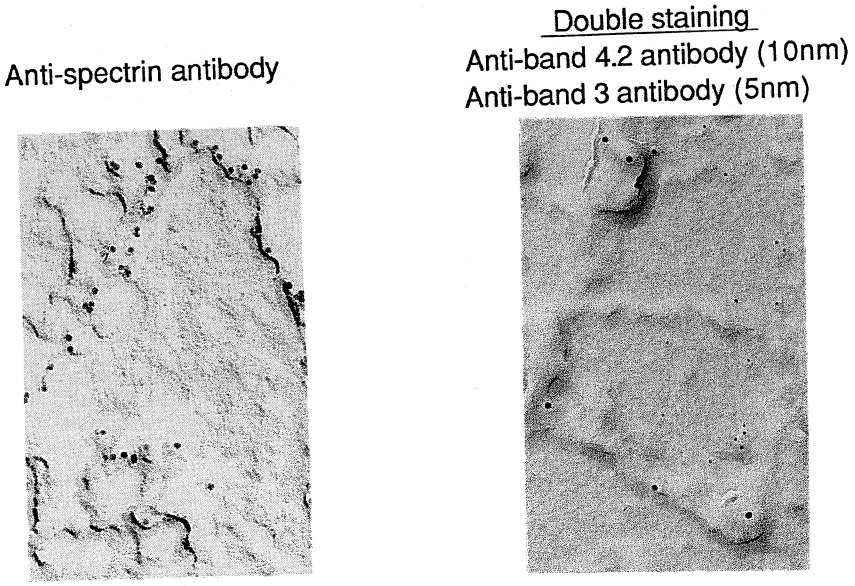

Fig 4. Protein 4.2 is co-localized with cytoskeletal network.
Red cell membrane ghosts were gently treated with 0.1 mM NaCl at 37°C for 10 minutes, and immunoelectron microscopy was performed with antispectrin antibody (the left case), or with anti-protein 4.2 antibody with the immunogold particles of 10 nm in diameter and anti-band 3 antibody with those of 5 nm in diameter (the right case). Anti-spectrin antibody clearly identified the localization of spectrins on the cytoskeletal network. The double staining method of immunoelectron microscopy indicated that protein 4.2 was localized only on the cytoskeletal network.

integral protein in early erythroblasts. In contrast, there has been little precise description of the expression of protein 4.2 during erythroid maturation, although indirect evidence has been found in the peripheral red cells of a mouse given a single injection of ^{35}S-methionine, by which protein 4.1 and 4.2 appeared to be synthesized at the same level throughout erythropoiesis.[87]

In a study using phase liquid culture method, in which normal peripheral mononuclear cells containing the burst-forming unit-erythroid (BFU-E) were utilized,[88] sequential expression of membrane proteins during the maturation of human erythroid precursors has recently been shown. Using this method, spectrin was detected at the early stage of the second phase (probably earlier than proerythroblasts), followed by band 3 in early erythroid cells. Protein 4.1 was detected on day 9 of the second phase of this method, corresponding to protein 4.1b, while peripheral blood cells exhibited both protein 4.1a and 4.1b. Protein 4.2, however, was barely recognized even on these days of the culture. It first appeared on day 10, when spectrin and band 3 were definitely expressed, as shown in Figure 5. The content of protein 4.2 in the cultured erythroid cells on day 10 was approximately $5\pm4\%$ of that in the same number of peripheral red cells.[89] The extent of protein 4.2 expression increased sequentially, to $29\pm10\%$ on day 14, and $37\pm18\%$ on day 16, when, in addition to a major band (72 kD) of protein 4.2, two more faint bands were detected by Western blotting with antiprotein 4.2 antibody, probably as isoforms of protein 4.2. Flow cytometric analyses confirmed these results.

At the mRNA level, no protein 4.2 mRNA was detected in normal

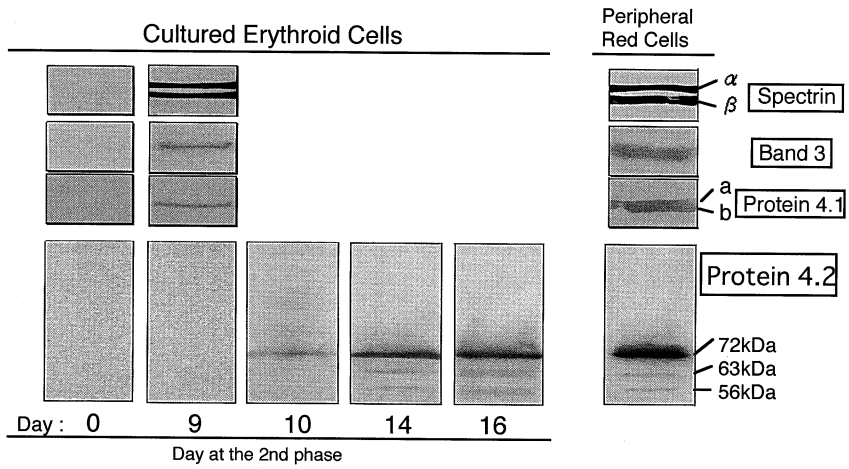

Fig 5. Expression of spectrin, band 3, protein 4.1, and protein 4.2 during maturation of erythroid cells by the two-phase liquid culture method.

The whole lysates of erythroid cells cultured by the two-phase liquid culture method were subjected to regular Western blotting with anti-human antibodies on SDS-PAGE. α-Spectrin, β-spectrin, band 3, and protein 4.1 were detected on day 9 of the second phase in the cultured cells. This protein 4.1 corresponded to protein 4.1b, while peripheral blood cells (PB) exhibited both protein 4.1a and 4.1b. On this day, protein 4.2 had still not been expressed. Protein 4.2 was first detected on day 10, and gradually increased in amount. In protein 4.2, an major 72 kDa peptide as a wild form and two minor bands (63 and 56 kDas) in trace amounts were detected.[89]

cultured erythroid cells even by RT-PCR on day 0 of the first and second phases of this culture method.[89] After day 3, protein 4.2 mRNA was detected by RT-PCR, as shown in Figure 6. Using Southern blot analysis, seven bands of protein 4.2 RT-PCR products were detected on day 3 and 8 of the second phase by autoradiography, as shown in Figure 7. These seven bands were also detected in reticulocytes in peripheral blood, but there were none in mononuclear cells present in peripheral blood. The sizes of the detected products were (I) 1052, (II) 986, (III) 915, (IV) 889, (V) 813, (VI) 709, and (VII) 595 base pairs, which appeared to correspond to the theoretical sizes deduced from the protein 4.2 sequence, that is: 1070, 980, 965, 875, 836, 746 or 712, and 641 base pairs, respectively. These findings were also confirmed by silver stain. At the cDNA level, these products correspond to (Type I) 2163, (II) 2073, (III) 2058, (IV) 1968, (V) 1929, (VI) 1839, (VI') 1824, and (VII) 1734 base pairs, which are isoforms deduced directly by gene sequences. The largest isoforms (Type I) of protein 4.2 were produced by reading the full length of the protein 4.2 gene. A wild type of the 72 kD (II) was obtained by skipping of 90 nucleotides in exon 1. By sequencing, other isoforms were also identified to be obtained by skipping only exon 5 (III); by combined skipping of 90 nucleotides in exon 1 and exon 5 (IV); by skipping exon 3 (V); by combined skipping of 90 nucleotides in exon 1 and exon 3 (VI); by skipping exon 3 and 5 (VI'); and by combined skipping of 90 nucleotides in exon 1, exon 3, and exon 5 (VII).[89]

These findings clearly indicate that, sequentially, spectrin was expressed in very early erythroblasts, followed by band 3 and protein 4.1. Then protein 4.2

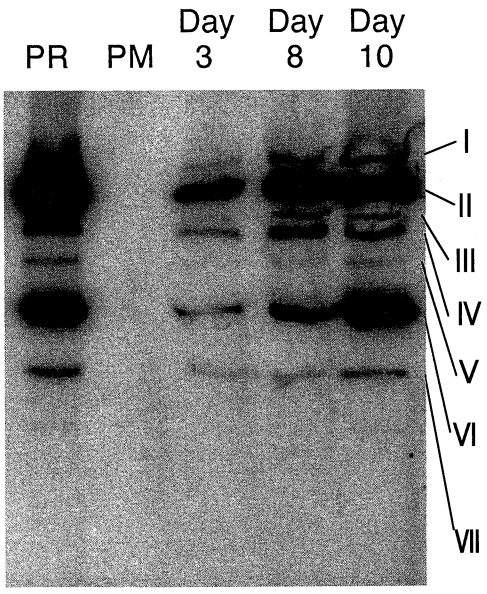

Fig 6. Expression of erythroid protein 4.2 cDNA isoforms during maturation of erythroid cells by the two-phase liquid culture method.

Protein 4.2 mRNA was amplified by RT-PCR with the primers A/G and H/G (see reference 89) with the Gene Amp RNA Kit to search for cDNA isoforms. The size of the probe was 327 bp (from exon 5 to exon 7) with the primers H/G. Twenty nanograms of the DNA probe were labeled with random primer with 50 μCi [^{32}P] CTP. Southern blot analysis was performed by the method of Winter *et al*. The gene products were tranferred to nylon membrane by the electrotransfer method. After PCR products were hybridized with the internal probe labeled with ^{32}P, autoradiographs were developed. Seven bands of protein 4.2 RT-PCR products were detected on days 3, 8 and 10 of the second phase of the two-phase liquid culture method by autoradiography. These seven bands were also detected in peripheral reticulocytes (PR), but not in mononuclear cells present in peripheral blood (PM).[89]

was expressed in very late erythroblasts or around the stage of reticulocytes. Thus, the expression of protein 4.2 appears to be the latest event among various membrane proteins,[89] as summarized in Figure 8.

In disease states, several isoforms of protein 4.2 are known to be present in human erythroid cells; i.e., 72 kD, 74 kD, 67 kD, and others.[33,35,36,60,61] Even in normal red cells, at least three isoforms of protein 4.2 have been detected by Western blotting with the Protein-A method.[89] The significance of the appearance of these isoforms of protein 4.2 isoforms is unknown. The 72 kD and 74 kD peptides were detected in trace amounts by Western blotting in the red cells of protein 4.2 deficiency of the Nippon type (a homozygote of codon 142 GCT → ACT),[31,33] and the 74 kD peptide in heterozygotes of protein 4.2 Shiga (codon 317 CGC → TGC)[35] and heterozygotes of protein 4.2 Komatsu (codon 175 GAT → TAT).[36] Variants of protein 4.2, in which 72 and 74 kD peptides were equally or nearly equally expressed in red cells, have also been reported as a protein 4.2 doublet Kobe and a protein 4.2 doublet Nagano.[5,6] Therefore, the control mechanism of expression of protein 4.2 should be clarified with respect to the phenotypic expression of protein 4.2.

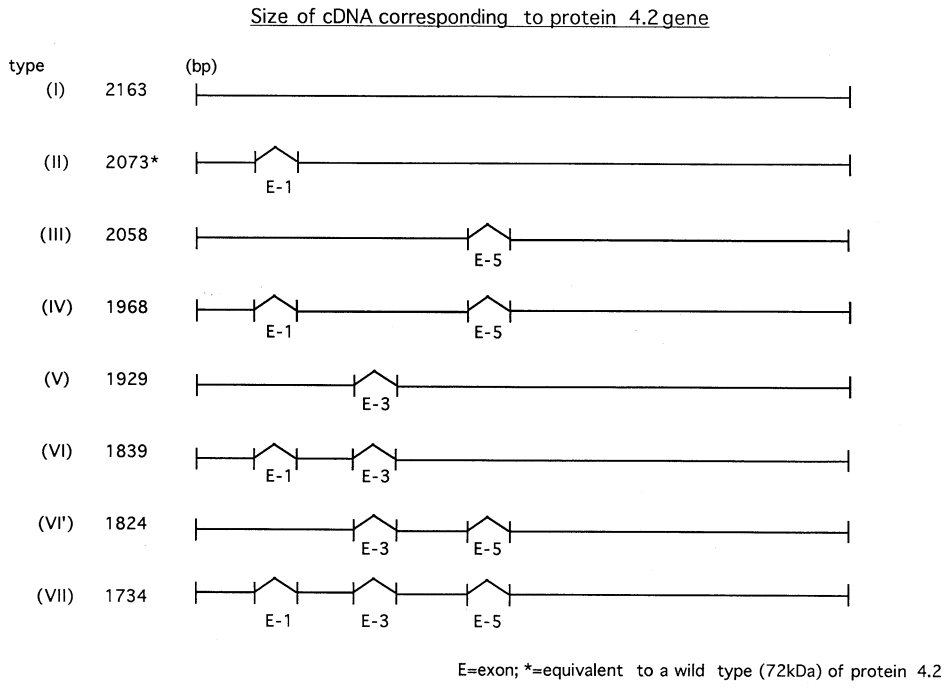

Fig 7. Presence of seven products of human erythroid protein 4.2 pre-mRNA or mature mRNA produced by various exon skippings.
Eight gene products of human erythroid protein 4.2 could be obtained, which were deduced directly by gene sequences. However, the expression of seven gene products among them were confirmed by Southern blot analysis (see Figure 6). The products VI and VI' were difficult to separate due to a minute difference of their product sizes. The results are schematically shown. E=exon; *=equivalent to a wild type (72 kDa) of protein 4.2.

2. Developmental expression of mouse red cell protein 4.2 mRNA in erythroid and non-erythroid tissues

It has been shown that erythropoiesis in mouse embryos is initiated at the yolk sac (E7.5-11.5 days) as a primitive erythropoiesis, by which nucleated erythroblasts are produced from the blood islets. At E12.5-16.5 days, the liver becomes the major erythropoietic organ producing erythroblasts, which can be differentiated to enucleated red cells in the peripheral blood, as a definitive erythropoiesis. After E16.5 days, the hematopoietic cells in the liver begin to recede in hematopoietic function and their proliferation decreases. Around and after birth, the spleen and the bone marrow become the major hematopoietic organs in mice.[27]

Expression of the mouse protein 4.2 gene, the protein 4.2 mRNA, and protein 4.2 itself has recently been studied by Northern blot analysis and by in situ hybridization during mouse embryogenesis.[27] To assess expression of the protein 4.2 gene, poly A^+RNAs from various adult mouse tissues were analyzed by Northern blot analysis using a 714-base pair mouse protein 4.2 cDNA fragment containing the 3' portion of the protein 4.2-coding region as the probe. A single 3.5 kb protein 4.2 transcript was detected at a relatively high level in the spleen. Little or no protein 4.2 hybridization was observed in

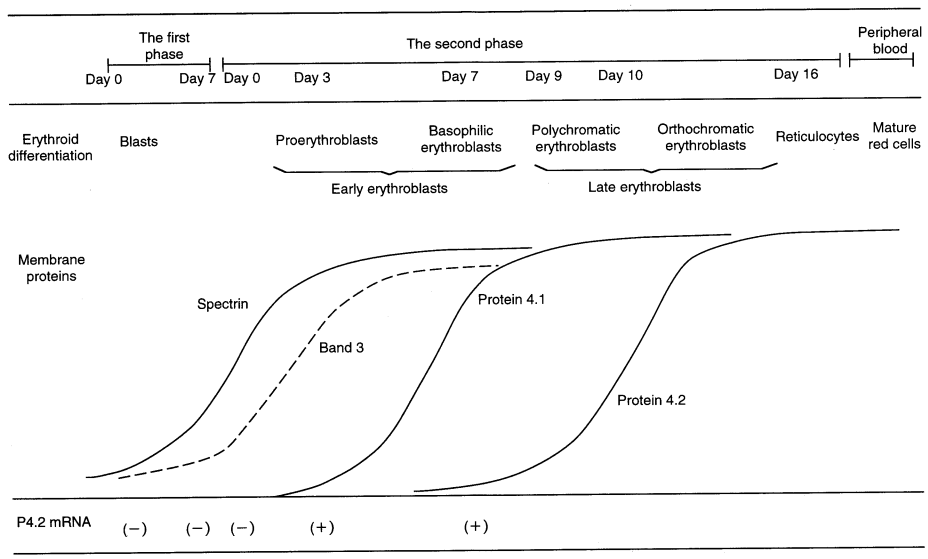

Fig 8. Schematical demonstration of sequential expression of red cell membrane proteins studied by the two phase liquid culture method with BFU-E from the peripheral blood.
Spectrins appeared to be expressed in early erythroblasts, and band 3 to be expressed probably also nearly at the same stage, followed by protein 4.1. However, protein 4.2 was not expressed with the stage of late erythroblasts. Even the protein 4.2 mRNA was not expressed at day 0 of the first phase which is equivalent to the stage of blasts, and remained unexpressed at day 0 of the first phase and also at day 0 of the second phase. The mRNA of protein 4.2 was first detected at day 3 of the second phase. Therefore, protein 4.2 appears to be a membrane protein, which is expressed at last among various red cell membrane proteins.[89]

other tissues examined (brain, lung, liver, skeletal muscle, kidney, or testis).[27]

At different stages of development, no protein 4.2 hybridization was detected in mRNAs from E6.5 embryos. The expected 3.5 kb protein 4.2 mRNA was first detected in E7.5 embryos, and its intensity increased in the embryos at later ages (E14.5 and E16.5). Protein 4.2-specific labeling was found to be localized in primitive erythroid cells. Starting from E12.5 days, there was a switch in the hematopoietic sites from the yolk sac to the fetal liver, where protein 4.2 expression was observed. By E14.5 days, the liver showed the strongest protein 4.2 hybridization signal among all organs. The protein 4.2 hybridization signal was greatly reduced in the liver after E16.5 days, and was almost undetectable after birth, when the spleen and bone marrow became the major hematopoietic sites. Significant protein 4.2 expression was seen in the emerging red pulp of the spleen.[27]

Throughout embryogenesis, no protein 4.2-specific labeling was observed in the lymphocytic organs (the thymus, the white pulp and germinal centers of the spleen), lymphocytes, plasma cells, macrophages, or megakaryocytes, but was detected in erythroid cells, and particularly at high intensity in areas with more mature erythroid cells.[27] Even in the bone marrow, no protein 4.2 hybridization signal was seen in cells of nonerythroid lineage, including megakaryocytes.[27] The protein 4.2 message was specifically expressed in cells of erythroid lineage in postnatal hematopoietic organs.

Immunoreactive forms of the red cell protein 4.2 have been reported in nonerythroid tissues such as brain, kidney, heart, liver, platelets, human endothelial cells, HT-29 human colon adenocarcinoma cells, T-84 human colon carcinoma cells, SK-N-MC human neuroblastoma cells, HeLa human cervical epithelial carcinoma cells, and CV-1 monkey kidney fibroblast cells, a Sf9 insect cell line.[4,22,23,26] The most recent work detected no protein 4.2 message in nonerythroid organs or megakaryocytes by Northern blot analysis.[27] Therefore, the immunoreactive forms of protein 4.2 detected in nonerythroid tissues or cells, which have been reported previously,[4,22,23,26] do not appear to be protein 4.2 isoforms, although they may be protein 4.2-related proteins. Further characterization of the molecular nature of these protein 4.2-immunoreactive analogs is needed to identify them.

The protein 4.2 gene in mice is mapped to mouse chromosome 2.[28] Its chromosomal location was found to be near the mouse pallid (pa) mutation.[4,22,28] Pallid is one of several independent mouse mutations that are models of the platelet storage pool disease.[29,30] With Southern blot and Northern blot analyses, it has been suggested that this pallid mutation (pa) in mice is a mutation in the protein 4.2 gene.[4,22,28] Under the above-mentioned assumption, the name of "pallidin" has been proposed for protein 4.2.[4] However, human patients with protein 4.2 deficiency have not shown any abnormalities of the platelets such as the platelet storage disease, observed with the pallid mutation (pa/pa), but have demonstrated uncompensated hemolytic anemia.[5,6,31–39] The mutant (pa/pa) mice did not have the increased hemolysis and spherocytosis seen in human patients.[29,30] Recently, the proposal that pallid is a mutation in the protein 4.2 (Epb 4.2) gene has been weakened by the fact that the gene encoding protein 4.2 is distinct from the mouse platelet storage pool deficiency mutation pallid by themselves after their thorough investigations.[25] Therefore, the name "pallidin" is inappropriate and misleading in describing protein 4.2.

It also has been reported that the red cell protein 4.2 gene (Epb 4.2) was present in normal platelets and absent from protein 4.2-null mice produced by the targeted protein 4.2 gene, although normal platelet counts with normal platelet functions have been observed in protein 4.2-null mice.[71] In this study, the immunoblotting method was utilized to detect the presence of protein 4.2 in normal mice.[71] The discrepancy between this finding and those by other investigators[27] should be clarified in the near future.

Disease states of protein 4.2 in human red cell membranes

Protein 4.2 abnormalities of congenital origin are classified into two groups; i.e., (1) deficiency (reduced content) of protein 4.2, and (2) protein 4.2 variants with normal or nearly normal protein 4.2 content.[5,6]

The first category of protein 4.2 deficiencies is further divided into two subgroups; i.e., (1) complete deficiency, and (2) partial deficiency.[5,6]

1. Complete protein 4.2 deficiency

i) Clinical hematology

Although a slight variation in clinical observations exists among the reported cases with total protein 4.2 deficiency,[31–39] the characteristic clinical

features are moderate, uncompensated hemolysis with moderate reticulocytosis, and increased indirect bilirubin. The hemolysis usually responds to splenectomy.[5,6] Regarding cell hydration, the mean corpuscular hemoglobin concentration (MCHC) in protein 4.2 deficiency is minimally elevated, in comparison with typical cases of classical, autosomal-dominantly inherited HS, in which substantial microspherocytosis and increased MCHC are usual.[5,6,31-39] In 17 cases with protein 4.2 Nippon,[90] who were homozygotes of a missense mutation (codon 142 GCT → ACT) of the protein 4.2 gene, red cell counts were $3.78 \pm 0.41 \times 10^6/\mu L$, MCHC 34.8 ± 0.1 g/dL, and reticulocytes $6.2 \pm 2.5\%$. A homozygote with protein 4.2 Komatsu, who carried a missense mutation (codon 175 GAT → TAT) of the protein 4.2 gene, demonstrated lower red cell counts ($3.3 \times 10^6/\mu L$), and higher MCHC (35.2 g/dL) and reticulocytes (12.4%).[36] In a compound heterozygote with protein 4.2 Nippon and protein 4.2 Shiga of a missense mutation (codon 317 CGC → TGC) of the protein 4.2 gene, red cell counts were $4.36 \times 10^6/\mu L$, MCHC 37.4 g/dL, and reticulocytes 4.0%.[35]

Red cell morphology varies among the patients with protein 4.2 deficiency. Protein 4.2 Nippon and protein 4.2 Komatsu patients consistently demonstrate ovalostomatocytosis, which is characterized by the presence of elliptic cells (not exceeding 20%) and stomatocytic changes superimposed on both discoid and elliptocytic red cells.[5,6,33,36,67,90] Microspherocytosis is only minimal. Instead, red cell morphology in protein 4.2 Shiga is characterized by microspherocytosis, which can hardly be differentiated from that of classical hereditary spherocytosis (HS).[35,67,90] Other patients with protein 4.2 deficiency have been reported as suffering from HS or the like in their red cell morphology.

The indirect bilirubin level was 1.8 ± 0.9 mg/dL in the reported patiens.[5,6,33,35,36,67,90] The osmotic fragility of the patients' red cells was consistently increased.[31-39,90]

ii) Red cell membrane proteins

Protein 4.2 content was completely or nearly completely missing in the red cells of these patients, when studied by the SDS-PAGE with Coomassie blue staining and also by the Western blot analysis with anti-protein 4.2 antibody,[5,6,31-39,90] as shown in Table 2. In the Nippon type, trace amounts of the 72 and 74 kD peptides were detected by immunoblotting, when an excess amount (50 μg) of the ghost proteins was applied.[31,33] The amount of the 74 kD peptide was nearly identical to that of the 72 kD peptide. This 74 kD peptide was also detected in a trace amount by immunoblotting even in normal Japanese individuals who have the 142 GCT → ACT point mutation.[5,6,33] The incidence of the 74 kD peptide in a trace amount detected in normal Japanese subjects was approximately 3%. The 74 kD peptide was not detected in protein 4.2 Komatsu without the missense mutation of codon 142 GCT → ACT.[36] In contrast, it was present among the compound heterzygote patients with protein 4.2 Shiga[35] and protein 4.2 Nippon.[31,33]

In the protein 4.2 deficiency, the content of band 3 was reduced by 10 ~20% of the normal value. The contents of most other membrane proteins, including spectrin, ankyrin, and protein 4.1 were essentially unaffected.[5,6,31,33]

TABLE 2. Phenotypic Characteristics of Total Deficiency of Protein 4.2 in Human Red Cells

	Red cell morphology	Red cell membrane proteins			Incidence		
		P4.2 content	P4.2 isoforms	B3 content	Race	Kindred (No.)	Patients (No.)
1. P4.2 Nippon	OS	nearly null	72 and 74 kDs (trace)	normal	J	13	17
2. P4.2 Shiga	HS (MS)	nearly null	72 and 74 kDs (trace)	normal	J	1	2
3. P4.2 Komatsu	HS (MS)	none	none	normal	J	1	1
4. P4.2 Tozeur	HS	none	none	normal	T	1	2
5. P4.2 Lisboa	HS	none	none	normal	P	1	1
6. P4.2 Fukuoka	HS	none	none	normal	J	1	2
7. P4.2 Notame	HS	nearly null	72 and 74 kDs (trace)	normal	J	1	1
8. B3 Okinawa	HSt	none	none	decreased (−42%)	J	1	4

P4.2: protein 4.2, B3: band 3, OS: ovalostomatocytosis, HS: hereditary spherocytosis, MS: microspherocytosis, HSt: hereditary stomatocytosis, J: Japanese, T: Tunisian, and P: Portuguese.

iii) Red cell membrane lipids

The content of red cell membrane lipids appeared to be normal in relation to total lipid content, free cholesterol, total phospholipids and their subfractions in the seven unsplenectomized patients with protein 4.2 deficiency,[5,6,33] when studied by standard methods with thin-layer chromatography.[91,92]

In protein 4.2 deficiency (n=7), red cell free cholesterol (FC) content was 1204±59 $\mu g/10^{10}$ red cells, compared with 1,202±103 in normal controls (n=152) and 1,084±63 in unsplenectomized HS (n=15). The total phospholipid content was 2,548±260 in protein 4.2 deficiency, 2,604±241 in normal controls, and 2,302±169 in unsplenectomized HS; phosphatidylethanolamine (PE) 734±48, 802±79, and 668±53; phosphatidylserine (PS) and phosphatidylinositol (PI) 436±30, 367±36, and 349±21; phosphatidylcholine (PC) 688±51, 729±60, and 664±56; sphingomyelin (SM) 645±20, 663±67, and 589±45; lysophosphatidylcholine (L-PC) 47±15, 39±11, and 32±7, respectively.[5,6,33]

iv) Red cell deformability

The membrane deformability of protein 4.2 deficient red cells has been reported as normal when studied in a non-stressed condition by ektacytometry.[5,6,31,33] Under conditions with various stresses such as heat treatment, however, membrane deformability is markedly abnormal.[5,6,33] Freshly drawn intact red cells from protein 4.2-deficient patients were studied by ektacytometry using the method of Mohandas *et al.*[93] The red cell suspensions were subjected to heat treatment from 37°C up to 48°C, and examined under various shear stresses (0 - 3000 dyne/cm^2). Red cell deformability was expressed as a deformability index. Marked impairment of red cell deformability was observed in the protein 4.2-deficiency especially beyond 46°C.[5,6,33] The deformability index in protein 4.2 deficient red cells was markedly decreased to 88% of that of normal controls at 45°C, 80% at 46°C, 61% at 47°C, and 52% at 48°C. The extent of the abnormality in the protein 4.2-deficiency was strikingly different from that in

classical HS, in which no essential changes were observed under the same conditions, as was the case with normal controls,[5,6,33] as shown in Figure 9. This finding indicates that protein 4.2 deficient red cells with the 142 GCT → ACT point mutation possess characteristic physiochemical properties different from those of red cells of classical HS with normal protein 4.2 content. Under heat treatment up to 48°C, no marked changes in red cell shape (budding formation and other poikilocytic shapes) were observed in the deficient red cells.[5,6,33] The MCHC was also unchanged.[5,6,33] The contribution of spectrins to marked impairment of the cytoskeleton was evaluated by incubating 37°C extracted crude spectrin at various temperatures (0, 46 or 48°C) to study dimer-to-tetramer conversion by the method of Liu *et al.*[94] The association constants (K_a) of dimer-to-tetramer conversion and the contents of high molecular weight (HMW) spectrin were essentially normal in these deficient red cells.[33] In another experiment, tetramer-to-dimer conversion of spectrin was performed in the ghost membranes of the red cells, which were subjected to 4, 46 or 48°C for 10 min by the method of Liu *et al.*[94] Spectrin was extracted at 4°C, and the contents of dimer and of HMW were calculated yielding normal results in these deficient red cells.[33]

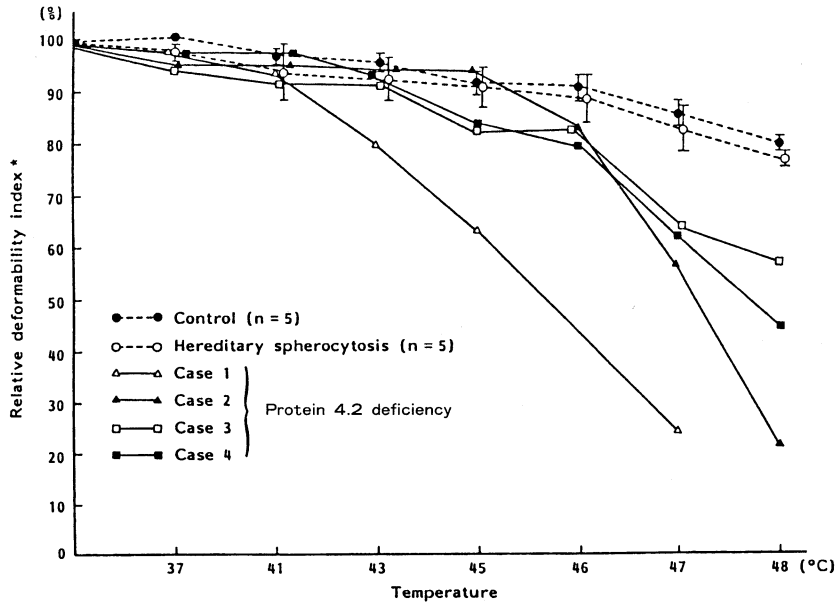

Fig 9. Markedly decreased membrane deformability of protein 4.2-deficient red cells under the heat treatment studied by ektacytometry.

Washed red cells were prepared with the 0.154 M Na/K phosphate buffer with glucose (150 mg/dL), and the hematocrit was adjusted to 10%. The red cells suspended in 290 mM (15%) dextran 40 solution were subjected to various temperatures (37° to 48°C), and were examined by ektacytometry under various shear stresses (0 to 330 dyne/cm²) by the method of Mohandas *et al.* Healthy subjects and the unsplenectomized patients with hereditary spherocytosis also were studied as controls. Red cell deformability was expressed as a deformability index.[5,6]

$$\text{Relative deformability index} = \frac{\text{DI of heat-treated red cells}}{\text{DI of non heat-treated red cells}} \times 100\ (\%)$$

Furthermore, spectrin was extracted at 4°C from red cell ghosts prepared from protein 4.2 deficient red cells treated with heat (4, 46 or 48°C). The contents of dimer and of HMW were normal. These results indicate that spectrin function itself appears to be nearly normal over a wide range of temperatures.

Red cell deformability is chiefly dependent on the functions of the cytoskeletons, which are composed of mostly spectrin in addition to protein 4.1 and actin. Therefore, if cytoskeletal functions are disrupted by the absence of protein 4.2, the red cells should lose their normal integrity in cell deformability. This was found to be so with protein 4.2 deficiency after heat treatment up to 48°C; the patient's red cells became markedly rigid losing their deformability, as determined by ektacytometry. Nevertheless, no bud formation was observed in red cell morphology under light microscopy, the MCHC was unchanged with normal cell water content, normal spectrin function was observed, and the mechanical stability of the Triton shells was normal.[5,6,33]

The cytoskeletal network appears to be linked normally to the lipid bilayer mostly via band 3 molecules in the presence of the normal amount of protein 4.2, which has been proven to be bound directly to spectrin molecules.[65] However, in the absence of protein 4.2, the cytoskeletal network barely remains nearly normal under an unstressed condition. Once these red cells are subjected to physicochemical stress, the network disassembles easily by losing its connection with the lipid bilayer. This seems to be a feasible interpretation for the abnormal red cell deformability in the protein 4.2-deficient red cells.

v) Biophysical characteristics

a) Extractability of band 3

The extractability of band 3 has been studied in protein 4.2-deficient red cells.[48] The patients' red cell ghosts were subjected to Triton X-100 (0.3-1.0%) at pH 8.0 at 4°C for 30 minutes. After the incubation, band 3 extracted from the red cell ghosts was examined on SDS-PAGE. The extractability of band 3 from the white ghosts was expressed as the ratio of the amount of band 3 remaining in the white ghosts/the amount of actin. The extractability in the protein 4.2-deficient red cells was 43.3±4.6% in 0.3% Triton X-100, 66.3±6.0% in 0.5% and 76.7±3.5% in 1.0%, compared with normal subjects (27.0±2.9%, 42.2±6.9% and 60.9±4.3%, respectively). Therefore, the extractability was enhanced significantly up to 60% of the normal control.[5,6,33]

These observations were also confirmed by other investigators.[65] Skeletons from protein 4.2-deficient red cells retained a greater fraction of band 3 protein than did skeletons from control cells after low-salt (0 mM NaCl) extraction. In contrast, equal fractions of band 3 protein were retained in skeletons from control and protein 4.2-deficient red cells after high-salt (150 mM NaCl) extraction. These results were consistent with the hypothesis that protein 4.2-deficient red cells are depleted specifically of band 3 molecules that are either unattached or bound with low affinity to the membrane skeleton.

Rybicki *et al*[66] extracted band 3 using the nonionic detergent octyl-β-glucoside, which extracts selectively band 3 that is not attached to the cytoskeleton and has been used to distinguish free band 3 from

cytoskeleton-bound band 3. One % octyl-β-glucoside respectively extracted 30% and 60% more band 3 from protein 4.2 Nippon and band 3 Montefiore, which demonstrated absence or substantial reduction of protein 4.2.

b) Binding of ankyrin to band 3

The inside-out vesicles (IOV) were prepared from protein 4.2-deficient red cells, and then ankyrin-deleted IOV was prepared. Ankyrin extracted from normal subjects was added to the ankyrin-deleted IOV, which was prepared from normal and protein 4.2-deficient red cells. The amount of ankyrin that was rebound to the patients' IOV was essentially normal.[5,6,33]

Other investigators noted that elutability of ankyrin was present in protein 4.2 deficiency, implying that protein 4.2 may stabilize ankyrin to bind to band 3.[31]

Red cell membranes in homozygous normoblastosis (nb/nb) mice have also been shown to be severely (up to 73%) protein 4.2 deficient.[69] Reconstitution of nb/nb IOVs with human red cell ankyrin restored ankyrin levels to 80% of that of normal IOVs, and increased binding of exogenously added human red cell protein 4.2 by 60%. Therefore, ankyrin may be required for normal associations of protein 4.2 with the red cell membrane.[69]

The role of ankyrin in the formation and stabilization of the spectrin-based skeletal meshwork and of band 3 oligomers was also studied. The results demonstrated that ankyrin was not required for the formation of a stable two-dimensional spectrin-based skeleton although it plays a major role in strengthening the attachment of the skeleton to the membrane bilayer. It is likely that ankyrin is required for the formation of stable band 3 tetramers.[70] The instability of ankyrin in protein 4.2 deficiency, as described above, may be due to a secondary phenomena, in which essentially normal band 3 molecules become unstable in protein 4.2 deficiency, resulting the instability of ankyrin.

c) Lateral mobilty of band 3

Band 3 lateral mobility is constrained in normal human red cell membranes by steric hindrance interactions, low affinity binding interactions, and high-affinity binding interactions.[65,95–97] Steric hindrance interactions between band 3 oligomers and the spectrin-based membrane skeleton put major constraint on the laterally mobile band 3 fraction, slowing the rate of band 3 lateral diffusion by approximately 50-fold compared with the predicted diffusion rate of free band 3 in membranes devoid of a functional membrane skeleton. The spectrin/band 3 ratio is the major determinant of the lateral diffusion rate of band 3.[65,95]

Fluorescence recovery after use of the photobleaching (FRAP) method has shown a shift in the lateral mobility of band 3 consistent with an increase in the mobile fraction of band 3 in membranes from individuals completely lacking protein 4.2.[5,6,33] The total recovery (mobile fraction), as shown in Figure 10, dramatically increased up to almost 100% with almost complete absence of the immobile component of band 3, as compared with normal subjects, in whom the mobile fraction was 0.43 ± 0.11 with a lateral diffusion coefficient of $(6.86 \pm 1.37) \cdot 10^{-11}$ cm^2S^{-1}, as reported by Tsuji *et al.*[96,97] Another common feature of the FRAP curves in these patients is that a nearly linear slow recovery component has appeared in addition to the fast recovery

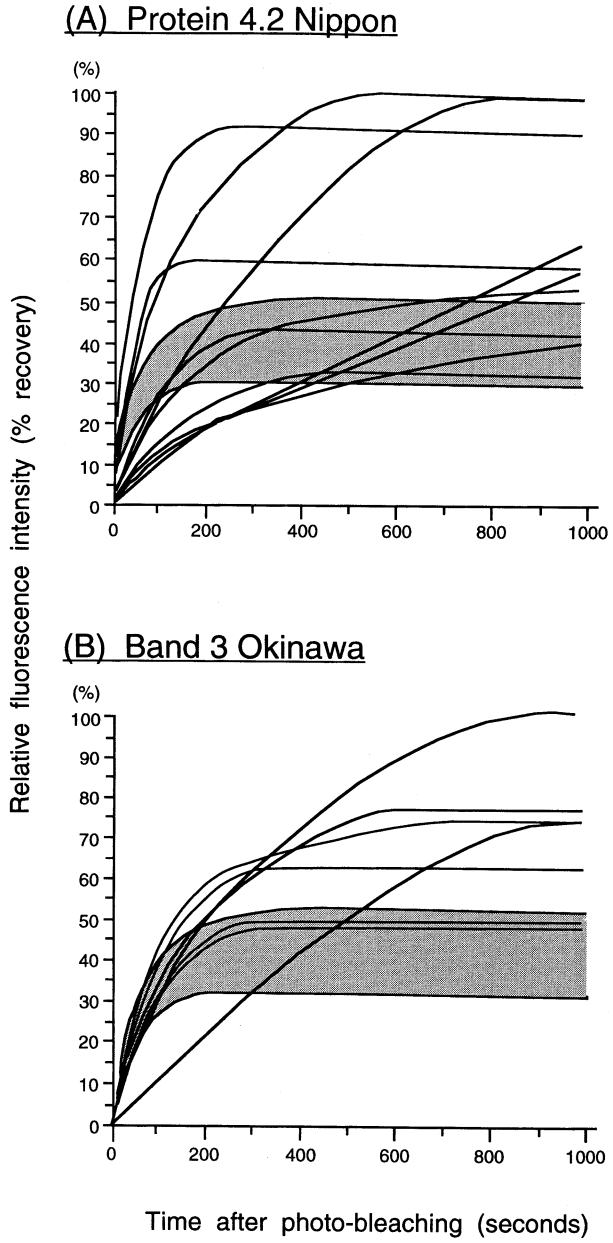

Fig 10. Lateral mobility of band 3 in red cell ghosts by fluorescence recovery after photobleaching (FRAP) method.

Labeling of band 3 with fluorescein isothiocyanate (FITC) and FRAP measurement of red cell ghosts in the buffer (5 mM Na_2HPO_4/NaH_2PO_4, and 10 mM NaCl (pH 7.6)) were performed by the method of Tsuji *et al.*[5] The measurements were performed in triplicate at 26°C using a 100×objective with 0.65 μm of a Gaussian beam radius. The results in normal control (n=20) are shown as a shaded area as based on mean values and standard deviation, and also those in protein 4.2 Nippon[5] (the upper panel: A) with total deficiency of protein 4.2 (n=10) and in band 3 Okinawa[61] (the lower panel: B) also with total deficiency of protein 4.2 (n=3) due to four missense mutations (K56E, P854L, G714R, and G130R) *in trans* on the band 3 gene. Details are described in text.

component observed in normal red cell ghosts.[5,6,33] These abnormal recovery curves suggest that the nearly linear slow recovery is not due to simple diffusion of band 3. Since the immobile component in normal ghosts is assigned to the band 3 that is bound to ankyrin,[95–97] the recovery curves suggest that, in protein 4.2 deficiency, the band 3 that was bound to ankyrin was mobile in the FRAP measurements. This, in turn, suggests that, in the absence of protein 4.2, dissociation of band 3 from ankyrin and reassociation of band 3 to ankyrin occurs frequently, and is detected as slow mobile fraction in FRAP measurements.[5,6,33] In contrast, in the presence of protein 4.2 in normal red cell ghosts, binding of band 3 to ankyrin is stable, dissociation does not occur during FRAP measurements, and the band 3 that is bound to ankyrin is detected as an immobile component.[5,6,33] In addition, the fraction of the fast mobile component of band 3 has been reported to be somewhat decreased in protein 4.2 deficient ghosts.[5,6,33] This change may be due to increased oligomerization of band 3, which was suggested by increased larger intramembrane particles, since it has been shown that increased oligomerization of band 3 decreases the mobile fraction of FRAP curves of band 3 in normal red cell ghosts.[95–97]

This observation has been further extended and reconfirmed by other investigators.[65] The lateral diffusion coefficient of band 3 in membranes of protein 4.2-deficient red cells was approximately two-fold greater than control values. In contrast, the lateral mobility of neither glycophorins nor a fluorescent phospholipid analog was altered in protein 4.2-deficient red cells. The increase in the band 3 lateral diffusion coefficient suggests that low-affinity binding interactions are significantly perturbed in protein 4.2-deficient red cells, and it is likely that the absence of protein 4.2 results directly in a decreased number of low-affinity binding sites for band 3 on the membrane skeleton.[65]

d) Rotational mobility of band 3

Band 3 rotational mobility is constrained in normal red cell membranes by low-affinity and high-affinity binding interactions.[65,95,97,98] The rotationally immobile band 3 fraction apparently represents individual band 3 molecules bound with high affinity to ankyrin. The rapidly rotating band 3 fraction consists of dimers, tetramers, and higher order oligomers of band 3 that are free from rotational constraints other than the viscosity of the lipid bilayer.[99] The slowly rotating band 3 fraction is less well-defined. Rotational constraints applied by low-affinity binding interactions between ankyrin-linked band 3 and other band 3 molecules, and between the cytoplasmic domain of band 3 and membrane skeletal proteins (ankyrin, protein 4.1 and protein 4.2) have been invoked.[100] Stenic hindrance interactions are not important in constraining band 3 rotational mobility.

The rotational mobility of band 3 in protein 4.2-deficient red cells has been studied by three groups.[65,66,101]

Golan *et al*[65] reported that, compared to control red cells, protein 4.2-deficient red cells manifested a significant shift from slowly rotating and rotationally immobile populations of band 3 to a rapidly rotating population, consistent with the interpretation that low-affinity binding sites for band 3 are decreased on the membrane skeleton of protein 4.2-deficient red cells. A significant increase was also noted in the correlation time of the rapidly

rotating band 3 population in protein 4.2-deficient red cells compared with control red cells. These results indicate that there is a shift from a lower to a higher order state of band 3 oligomerization in protein 4.2-deficient red cells, and that free band 3 dimers are lost preferentially in these red cells, leading to a larger average size of intramembrane particles visualized by freeze fracture electron microscopy.[5,33,67] These results suggest that protein 4.2-deficiency acts primarily to destabilize a fraction of band 3 molecules, resulting in loss of band 3 and possibly of the membrane.

The same observations were made by Rybicki *et al.*[66] Band 3 in both protein 4.2 Nippon (>99% protein 4.2-deficient) and band 3 Montefiore (~88% protein 4.2-deficient) ghost membranes showed an increased rotational freedom as compared with band 3 in control ghosts. The primary effect was a shift from slower-rotating and immobile fractions to more mobile, faster decay terms for these two phenotypes of protein 4.2-deficiency.

Contrary to these two reports,[65,66] Wyatt and Cheny[101] concluded that protein 4.2 had no effect on the rotational mobility of band 3. Possible reasons for these discrepancies may be a difference in the experimental conditions. In the cases of Wyatt and Cherry,[101] protein 4.2 was not completely depleted, whereas, in the two other reports,[65,66] protein 4.2 was almost totally absent.

vi) Membrane transport

a) Sodium transport

Sodium transport was examined in washed red cells with 0.154 M Na/K phosphate-buffered saline solution (pH 7.4) with glucose (250 mg/dL).[5,6,33] After incubation of the red cells with ^{22}Na at 37°C for two hours, the radioactivity of the ^{22}Na remaining in the incubated red cells was counted (sodium influx). Na efflux was determined by incubating the ^{22}Na-labeled red cells at 37°C for two more hours in the presence or absence of 1.08 mM ouabain in buffer not containing ^{22}Na. The extent of Na efflux was calculated from the radioactivities in red cells before and afier incubation. Red cell sodium and potassium were determined by flame photometry.

Sodium influx was moderately increased in the protein 4.2-deficient red cells (1.80±0.32 mM/L red cells/hour) compared with normal subjects (1.29±0.14). Under the same condition, Na influx was respectively 2.35±0.45 and 1.96±0.34 before and afier splenectomy in patients with hereditary spherocyiosis (HS).[5,6,33]

Total sodium efflux was markedly increased in the protein 4.2-deficient red cells (7.2±1.1 mM/L red cells/hour) compared with normal subjects (2.4±0.5). The increment was much more striking than that in the conditions before (4.4±1.1) and after (3.6±0.8) splenectomy in classical HS. Na efflux in the protein 4.2-deficiency was increased in both the ouabain-sensitive and ouabain-insensitive sodiuln efflux.[5,6,33]

Red cell sodium [Na] content was significantly elevated (15±4 mM) as compared with normal subjects (10±4), the same as it was in unsplenectomized HS patients (16±7). Red cell potassium [K] content was diminished (81±8 mM) as compared with normal subjects (90±5), same as it was in unsplenectomized HS patients (82±9) with overt hemolysis.[5,6,33]

Ouabain-sensitive Na efflux as Na/K pump in the red cells of protein 4.2

-deficiency has recently been shown to be increased (21.4±1.5 mmol/kg Hb× hour), compared with 15.2±1.9 in normal controls.[102] Bumetanide-sensitive Na efflux and bumetanide-sensitive K efflux, and Na/Li exchange, as Na/K/2 Cl cotransport, were basically normal.[102] Volume-chloride K efflux as K/Cl cotransport was 3.6±0.3 mmol/kgHb×hour in protein 4.2-deficiency, compared with 6.5±1.2 in normal subjects.[102] Overall Na influx and K efflux as membrane passive permeability were 33.1±4,1 and 7.2±2.3 mmol/kgHb×hour in protein 4.2 deficiency, compared with 15.4±2.7 and 2.1±0.2 in normal subjects.[102] Red cell sodium content (38.1±2.3 mmol/kgHb) was increased and potassium content (230±6 mmol/kgHb) were decreased in protein 4.2-deficiency, compared with Na (27.2±2.3) and K (287±10) in normal subjects.[102] Increased membrane passive permeability to cations was observed to the same extent among HS with band 3 deficiency, with ankyrin and spectrin deficiency, or with protein 4.2-deficiency,[102] independent of a specific membrane protein defect. Therefore, it may be speculated that cytoskeletal dysfunction *per se* may alter the permeability barrier of the red cell membrane.

The above-mentioned interpretation is supported by increased cation membrane permeability, which has been reported in various spherocytic mouse red cells.[103] Red cell sodium contents in homozygous sph^{ha}/sph^{ha}, sph/sph, and nb/nb mice were 30.1±0.9, 28.9±0.3, and 26.9±1.5 mmol/L red cells, respectively, compared with normal subjects (11.3±0.7).[103] Red cell potassium contents were 102±2.6, 101±7.8, and 97.4±3.0, compared with normal subjects (123±10).[103] It has been found that sph/sph exhibit only a trace of spectrin α chain with defective transcription, processing or stability of α-spectrin mRNA, that sph^{ha}/sph^{ha} apparently produces an unstable α chain with 20% to 30% of the normal spectrin complement and somewhat more β-spectrin than α-spectrin present, and that nb/nb results in reduced levels ankyrin mRNA with almost complete absence of the 210 kD protein in red cells and about a 50% decrease in spectrin, presumably secondary to loss of spectrin binding sites.[103] Sodium uptake by red cells was 14.8±1.6, 15.4±3.3, and 14.7±3.1 mmol/L red cells/hour in sph^{ha}/sph^{ha}, sph/sph, and nb/nb mutants, compared with normal subjects (3.9±1.0). Potassium loss from red cells was 17.0±4.0, 15.0±3.8, and 14.1±2.6 in sph^{ha}/sph^{ha}, sph/sph, and nb/nb, compared with normal subjects (6.0±2.1).[103] The red cells of these mutant mice with dysfunctional membrane skeletons have increased passive permeability to monovalent cations, suggesting that the membrane skeleton may be critical for maintenance of the membrane permeability barrier.

In the red cells of human protein 4.2-deficiency, a marked derangement of the cytoskeletal network has been verified by electron microscopy studies.[33,67] The cation transport abnormalities may be produced by an abnormal cytoskeletal network due to total absence of protein 4.2.

b) Anion transport

Red cell anion exchange has been shown to be sensitive to inhibition by stilbenedisulfonates.[104] Band 3 has two distinct domains; i.e., the cytoplasmic domain and the transmembrane domain.[105–107] The 55 kD transmembrane domain contains an anion-transporting site and can mediate anion exchange independently of the cytoplasmic domain. The cytoplasmic domain also has a distinct binding site for protein 4.2.[8] Increasing amounts of protein 4.2

complexed with band 3 have been shown to cause a decrease in band 3 mediated anion transport.[108] The inhibitory effect of protein 4.2 on band 3 mediated anion transport appears to be specific. The specific interaction of protein 4.2 with the cytoplasmic domain of band 3 causes reduction of its anion transport capacity. Protein 4.2 appears to have a possible heterotropic allosteric modulator of band 3 anion transport.[108]

In fact, a marked increase in di-isothiocyano-dehydrostilbene disulphonate (H_2DIDS)-sensitive sulfate influx has been observed in the red cells of protein 4.2 deficiency, while no remarkable changes in maximal inhibitory H_2DIDS concentration between in normal subjects and in protein 4.2 deficient patients have been evident.[102] Maximal sulfate influx in protein 4.2 deficiency ranged between 19.8 and 47.4 mmol sulfate/10^{13} cells$\times$hour compared with normal subjects (11.4-14.1). Maximal inhibitory H_2DIDS concentration in protein 4.2 -deficiency was from 2.0 to 2.5 μM, compared with normal controls (2.5 to 3.0). Therefore, the ratio between sulfate flux and the maximal inhibitory H_2DIDS concentration ranged from 8.6 to 20.6, in protein 4.2-deficiency compared with normal subjects (4.3 to 4.7), indicating a fourfold increase in activity of the anion transport by band 3 molecules.[102] Therefore protein 4.2 may act as a negative modulator of band 3-mediated anion transport.

However, another study has reported that anion transport in protein 4.2-deficient red cells was normal, and that protein 4.2 did not appear to be required for band 3 anion transport activity.[66] In this study, the calculated maximal rate (Vmax) and Km from the Michaelis-Menton equation in protein 4.2 Nippon were 0.24$\times$0.02 μmol/mL packed red cells/minutes and 7.0$\pm$1.8 mmol/L, compared with 0.25$\pm$0.02 and 6.2$\pm$1.3 in normal subjects.[66] Therefore, the role of protein 4.2 in anion transport by band 3 still remains to be elucidated.

vii) Ultrastructure of red cell membrane in situ

Electron microscopic studies have been carried out to elucidate the abnormalities of red cell membrane ultrastructure *in situ* in total protein 4.2 deficiency.[5,33,36,67] For this purpose, three independent probands were selected; i.e., 1) a clinically most severely affected protein 4.2 Komatsu proband[36] with a 175 GAT $\rightarrow$ TAT mutation (in exon 4) with total absence of protein 4.2 protein, 2) a moderately severely affected protein 4.2 Nippon proband[33] with a 142 Ala $\rightarrow$ Thr mutation (in exon 3) with a nearly missing protein 4.2 protein, and 3) a protein 4.2 Shiga,[35] a compound heterozygote with a 317 Arg $\rightarrow$ Cys mutation (in exon 7) and 142 Ala $\rightarrow$ Thr (in exon 3) *in trans* with protein 4.2 protein in a trace amount, with rather mild clinical severity.

a) Abnormalities of intramembrane particles (IMPs)

Intact red cells were subjected to electron microscopic studies using the freeze fracture method. In normal subjects, the number of IMPs was 5,210$\pm$389/μm^2, of which approximately 80% were basically small (4-8 nm) in size.[67] In the red cells of protein 4.2-deficiency of the three types, on the other hand, the number of IMPs had decreased to 4,464$\pm$353/μm^2 in the Nippon type, 4,625$\pm$381 in protein 4.2 Shiga, and 2,975$\pm$310 in protein 4.2 Komatsu.[67] Therefore, the decrement in the number of IMPs was most marked in protein 4.2 Komatsu, as shown in Figure 11. The decreased number of IMPs

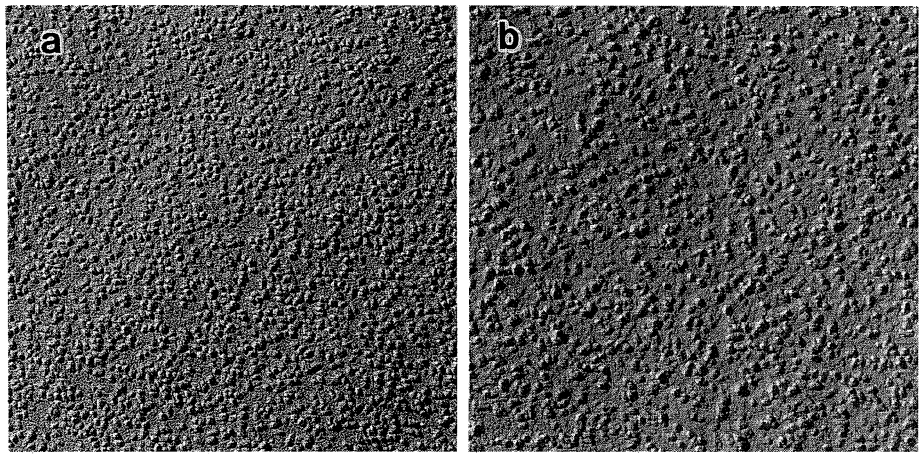

Fig 11. Electron micrographs of red cell membranes made by the freeze fracture method showing the distribution of intramembrane particles (IMPs) in normal subjects and the proband of protein 4.2 Komatsu with total deficiency of protein 4.2.
(a) Normal subject (the left panel). (b) Total protein 4.2 deficiency of the proband with protein 4.2 Komatsu.[36] A marked oligomerization of IMPs was noted in the proband.

appeared to be derived from a decreased number of IMPs of small size in association with an increased number of IMPs of medium (9-20 nm) and large size (>21 nm).[67] The condition of IMPs was found to be most affected in protein 4.2 Komatsu, as judged by a decreased number of IMPs with a marked shift to ones of a large size, probably indicating increased oligomerization of band 3 *in situ* (Figure 11). The extent of the abnormalities was less in protein 4.2 Nippon and the least in protein 4.2 Shiga, although these abnormalities were still demonstrated more than they were in normal controls.[67] It can be speculated that the total absence of protein 4.2 in protein 4.2 Komatsu may produce the most serious derangement in its interaction with band 3 molecules. There may be less serious effects with the other two mutations because of the presence of protein 4.2 even in a trace amount. Another speculation can be made that codon 175 of protein 4.2, as seen in protein 4.2 Komatsu, may be most important as the binding site of protein 4.2 to band 3, and that other codons (142 or 317) as in protein 4.2 Nippon or protein 4.2 Shiga may be important, but to a lesser extent.

The significant contribution of protein 4.2 to the biophysical properties of band 3 was proved by utilizing inside-out vesicles (IOVs) of normal controls and those of protein 4.2 deficiency.[67] In protein 4.2 deficiency, the distribution pattern of IMPs was totally deranged in IOVs, which were prepared from red cell ghosts of protein 4.2 Nippon, compared with those in the normal controls. When spectrins and membrane proteins other than band 3 were stripped from the IOVs at pH 11 in the normal controls,[7] this experimentally produced 4.2 deficiency demonstrated a markedly abnormal aggregation of band 3, which was the same as that in protein 4.2-deficient patients,[67] as shown in Figure 12. Therefore, it is clear that protein 4.2 should have the ability to maintain normal distribution of IMPs *in situ*, in which band 3 accounts for

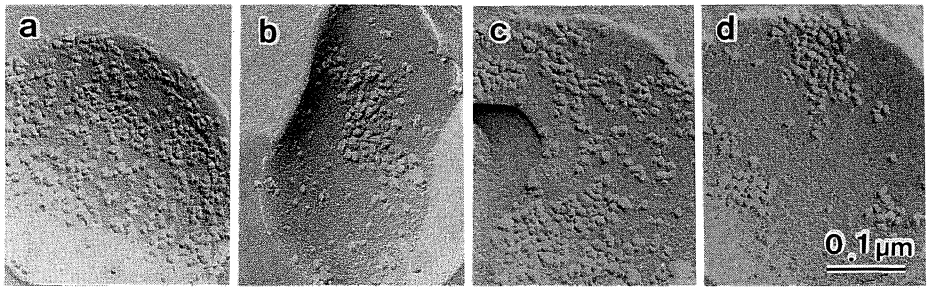

Fig 12. Electron micrographs of inside-out vesicles (IOVs) of red cell ghosts from protein 4.2 deficiency of the Nippon type.

Spectrin-depleted vesicles were prepared at pH 8.0 and were examined by electron microscopy using the freeze fracture method. a : Normal IOVs demonstrated an orderly distribution of IMPs. b : IOVs from protein 4.2 deficiency showed an uneven distribution of IMPs with marked aggregation (at pH 8.0). c : Normal IOVs subjected to pH 11 came to demonstrate marked aggregation of IMPs with uneven distribution (at pH 11). d : IOVs from protein 4.2 deficiency treated at pH 11. These findings were essentially the same as those in protein 4.2 deficiency (b at pH 8.0 and d at pH 11).[67]

approximately 80%.

b) Abnormalities of the cytoskeletal network

The cytoskeletal network has also been examined by electron microscopy using the quick-freeze deep-etching method (QFDE) method.[67] This procedure demonstrated that the filaments (mostly spectrins) of the intact cytoskeletal network in normal subjects were present in multistereotactic dimensions rather than in a single plane.[62,67,109] The filaments in the normal subjects were 48±9 nm in length and 7±1 nm in diameter, and appeared to be in a folded configuration. The cytoskeletal network in normal red cells showed a fairly uniform distribution of filamentous structures and also uniformity of apparent branchpoints of the filamentous elements in an essentially orderly fashion.[67] The cytoskeletal network in the normal subjects showed numerous basic units, resembling "cages", the number of which was 539±20/μm.[67] The "cage"-like structures consisted essentially of two major types of units; i.e., small (20-44 nm)- and medium (45-68 nm)-sized units as determined by the interdistance (or diameter) of the longer axis of each structure. In the normal subjects, two-thirds of these units were of small size (66±9%), and the remaining one-third were of medium size (30±6%). There were only a few large-sized units (4±1%) in the normal subjects.[67]

In contrast, in protein 4.2 deficiency, the uniform distribution of filamentous structures was lost, and apparent branchpoints of the filamentous elements were markedly distorted or disrupted.[67] Th extent of the abnormalities of the cytoskeletal network appeared most marked in protein 4.2 Komatsu, as shown in Figure 13, and less in protein 4.2 Nippon and protein 4.2 Shiga.[67] The abnormalities of the cytoskeletal network in protein 4.2 deficiencies were semiquantitated by counting the number of apparent cytoskeletal units still left as nearly recognizable and tolerable for these counting procedures. The number of these cytoskeletal units was markedly reduced in 4.2 Komatsu (195±38/μm^2), less in Nippon type (282±27/μm^2),

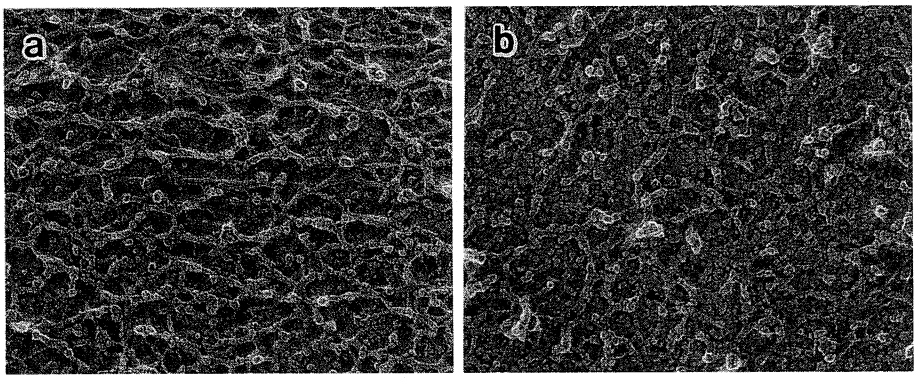

Fig 13. Electron micrographs of red cell membrane skeletons made by the quick-freeze deep-etching (QFDE) method. a (the left panel): normal control. b (the right panel): total deficiency of protein 4.2 of the proband with protein 4.2 Komatsu.[36]

and least in 4.2 Shiga ($339 \pm 35/\mu m^2$).[67]

The relative size distribution of these cytoskeletal units was also semiquantitated by measuring the interdistance (or diameter) of the longer axis in each unit.[67] In protein 4.2 deficiencies, the cytoskeletal units of basic small size (20-44 nm) were markedly reduced in 4.2 Komatsu ($25 \pm 4\%$), in Nippon type ($27 \pm 5\%$), and in 4.2 Shiga ($34 \pm 5\%$), compared with the normal controls ($66 \pm 9\%$). In their place, units of large size (69-92 nm) and of extra-large size (93-180 nm), which were essentially not present in the normal subjects, were tremendously increased in these protein 4.2 deficiencies.[67]

It is noteworthy that the cytoskeletal network was markedly disrupted in the protein 4.2 deficiencies, as evaluated by the number of cytoskeletal units left, the relative size distribution of these units, and the discontinuation of fibrous filaments.[67] The most striking abnormality of the cytoskeletal network was the disruption of the basic units of the network, as evaluated by the decreased number of these units in the protein 4.2 deficiencies. The number of the apparent units was decreased to one-third, especially in 4.2 Komatsu, as compared with that in the normal controls.[67] The contents of the cytoskeleton-related membrane proteins (spectrins, ankyrin, actin, and protein 4.1) were essentially normal on SDS-PAGE in these patients with protein 4.2 deficiencies.[33,35,36] Therefore the decreased number of apparent basic units of the cytoskeletal network should indicate marked instability of the network under a condition of absence of protein 4.2.[5,6,67] This interpretation is supported by findings regarding the relative size distribution of the cytoskeletal units, as judged by the interdistance (or diameter) of the longer axis of these units. In the protein 4.2 deficiencies, the units of small size (20-44 nm), which are the major structure (as 66% of the total units in number) under normal conditions, were reduced to only 25-34% of the total units, in association with a marked increment of large (69-92 nm) units and even of extra-large (93-180 nm)-sized units. The disruption was the most marked in protein 4.2 Komatsu, and less in protein 4.2 Nippon and protein 4.2 Shiga.[67]

c) Important role of protein 4.2 in stabilizing the cytoskeletal network by its binding to band 3

Under normal conditions, the cytoskeletal network is believed to be stabilized by binding to band 3 molecules tightly via ankyrin.[4,5,65,95–98] Two-thirds of band 3 is immobilized by this binding, but the other one-third is mobile and unfixed without binding to the cytoskeletal network.[4,5,65,95–98] In the absence of protein 4.2, the cytoskeletal network appeared to become extremely unstable due to the loss of the integrity of its basic small units, resulting in disruption of the interconnected structure of the cytoskeletal network.[5,65,67] Under this pathological condition with a markedly impaired cytoskeletal network, band 3 , two-thirds of which is normally connected with the cytoskeletal network mainly via ankyrin, should lose its binding to the network and become unfixed and mobile. It is known that free band 3 molecules tend to aggregate or cluster. The increased large sizes of the IMPS in the protein 4.2 deficiencies may be the result of aggregation and/or clustering of these increased mobile band 3 molecules, which were initially immobile band 3 bound to the cytoskeletal network.[5,6,65,67] The aggregated or clustered band 3 should naturally produce a decrease in the apparent number of IMPs.

It has also been shown that band 3 has binding properties to ankyrin, which binds to β-spectrin.[95–98] The bindings probably enhance the vertical stability of the cytoskeletal network. In the three proband with protein 4.2 deficiencies, the contents of the cytoskeletal proteins and of ankyrin were essentially normal.[33,35,36] Therefore, the stability of the cytoskeletal network should have been maintained normally in the presence of band 3 and ankyrin, independent of the presence or absence of protein 4.2, if protein 4.2 itself does not have its direct binding to the cytoskeletal network. However, in protein 4.2 deficiency, in which band 3 and ankyrin are present normally, marked instability of the cytoskeletal network was observed.[33,36,67] Therefore, it is very likely that protein 4.2 should have its direct binding to the cytoskeletal proteins, especially to spectrins, although the tertiary structure of the protein 4.2 molecule has not been elucidate.[65] A protein 42-deficient state should also produce aggregation or clustering of IMPs, probably by increasing free mobile band 3.

In summary, direct evidence of an impaired cytoskeletal network and of abnormal IMPS was shown in protein 4.2 deficiency by utilizing three probands with different mutations of the protein 4.2 gene. In the structure of protein 4.2, the aspartic acid at codon 175 may be one of the most important factors for maintaining its cellular function, along with alanine at codon 142 and arginine at codon 317. These results clearly indicate that protein 4.2 plays an important role in maintaining the integrity of normal IMPs and a normal cytoskeletal network in situ.

viii) Protein 4.2 gene mutations

Seven kinds of total protein 4.2 deficiency due to mutations of the protein 4.2 gene have been identified[32–39]; i.e., four missense mutations,[32,35,36,38] one frameshift mutation,[37] one nonsense mutation,[34] and one donor site mutation due to intronic substitution,[39] as summarized in Table 3. Therefore, missense mutations are predominant, especially allele protein 4.2 Nippon (142 GCT →

ACT : Ala → Thr),[32-34,36,39] which has been observed in 17 patients of 13 kindreds among 28 patients of 19 kindreds with complete protein 4.2 deficiency,[90] as shown in Table 2. These protein 4.2 gene mutations have been found mostly or nearly exclusively in the Japanese population ;i.e.. protein 4.2 Nippon,[32,33] protein 4.2 Shiga,[35] protein 4.2 Komatsu,[36] protein 4.2 Fukuoka,[34] and protein 4.2 Notame.[39] (Table 2) Only two mutations have been observed in the non-Japanese population ; i.e., protein 4.2 Tozeur in Tunisia (310 CGA → CAA)[38] and protein 4.2 Lisboa in Portugal (88 AAG GTG → AAG TG)[37] in addition to one Italian Caucasian patient who was a homozygous protein 4.2 Nippon.

TABLE 3. Genotypic Characteristics of Total Deficiency of Protein 4.2 in Human Red Cells

Mutated allele	Affected gene	Position of exon (E) of Intron (I)	Affected base	Mutated amino acid	Type of mutation	Abnormal allele
Allele P4.2 Lisboa	P4.2	E2	264 or 265 G deleted (AAG GTG → AAG TGG)	88 Lys-Val → Lys-Trp	deletion (frameshift)	homozygote
Allele P4.2 Fukuoka	P4.2	E3	357 TGG → TGA	119 Trp → Stop	missense (nonsense)	compound heterozygote with allele P4.2 Nippon
Allele P4.2 Nippon	P4.2	E3	424 GCT → ACT	142 Ala → Thr	missense	homozygote
Allele P4.2 Komatsu	P4.2	E4	523 GAT →TAT	175 Asp → Tyr	missense	homozygote
Allele P4.2 Notame	P4.2	I6	intron 6 donor site G → A	308 → frameshift	splicing (frameshift)	compound heterozygote with allele P4.2 Nippon
Allele P4.2 Tozeur	P4.2	E7	929 CGA → CAA	310 Arg → Gln	missense	homozygote
Allele P4.2 Shiga	P4.2	E7	949 CGC → TGC	317 Arg → Cys	missense	compound heterozygote with allele P4.2 Nippon
Allele B3 Okinawa	B3	E6	388 GGA → AGA (B3 Fukuoka)	130 Gly → Arg	missense	
	B3	E4	166 AAG → GAG (B3 Memphis I)	56 Lys → Glu	missense	compound heterozygote
		E17	2140 GGG → AGG (B3 Okinawa)	714 Gly → Arg	missense	
		E19	2561 CCG → CTG (B3 Diego)	854 Pro → Leu	missense	

Among these protein 4.2 gene mutations, the mutation of the Nippon type (142 GCT → ACT) is most important in the Japanese population,[5,6,90] because it is involved in homozygotes of protein 4.2 Nippon,[32,33] and also in compound heterozygotes of protein 4.2 Shiga with 317 (CGC → TGC),[35] protein 4.2 Fukuoka with 119 (TGG → TGA),[34] and protein 4.2 Notame with G → A at the intron 6 donor site.[39] (Table 3)

The mutations of the protein 4.2 gene appear to be clustered around exon 3 and at the 5' side of exon 7. Interestingly, no mutation has been reported from exon 8 to exon 13 at the C-terminus, as shown in Figure 14. Therefore, the regions around exons 3 and 7 could be biologically important as so-called "hot spots". An experiment with the targeted protein 4.2 gene for its knock-out mice has recently been designed based on these observations.[71]

Allele frequency of the Nippon type (142 GCT → ACT) appears to be around three percent in the normal Japanese population.[5,6]

Complete protein 4.2 deficiency appears to be transmitted by autosomal recessive inheritance, and most patients have been homozygotes or compound heterozygotes of missense mutations on the protein 4.2 gene.[32-39] Therefore, sole heterozygotes of these missense mutations have been asymptomatic with nearly normal protein 4.2 content in red cells.[5,6,33,90]

In two patients with protein 4.2 doublet Nagano, in which two protein 4.2 peptides of 72 kD and 74 kD were expressed in nearly equal amounts, the

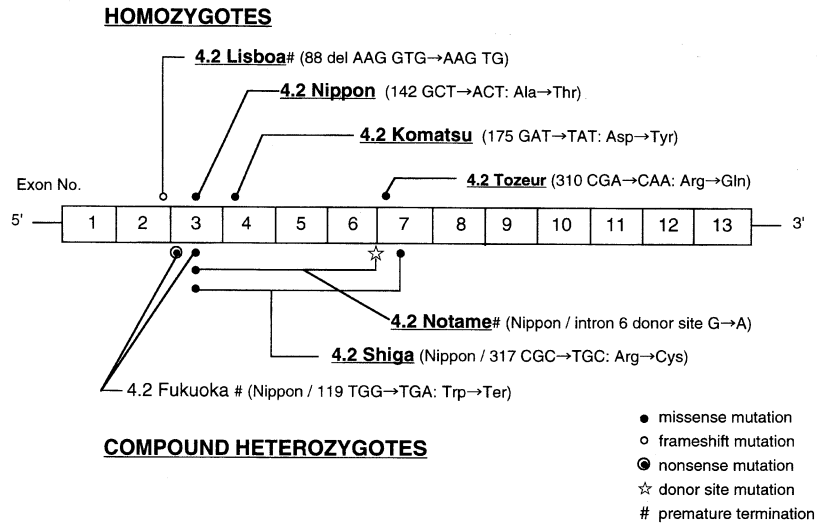

Fig 14. Mutations on the protein 4.2 gene with total deficiency of protein 4.2 in human red cells.

mutation of the protein 4.2 gene (488 CGT → CAT in exon 10) was linked to this abnormality by heterozygotes in the family members.[110]

ix) Band 3 gene mutations

Band 3 is one of a family of anion exchanger (AE) genes. AE1 is expressed in red cells and also in both mouse and human kidney as an alternative isoform that utilizes a downstream start codon and excludes exons 1 to 3.[111] These exons encode amino acids involved in membrane skeleton binding,[112] and recent evidence confirms that this function is lacking in the truncated kidney isoform.[113,114] AE2 is ubiquitously expressed but is especially prominent in the gastrointestinal tract and choroid plexus, while AE3 is expressed in brain neurons, the retina, heart, and kidney.[115] The membrane spanning domains of AE1, AE2, and AE3 are highly conserved and function in anion exchange. The cytoplasmic domains show less conservation. AE2 and AE3 have ~300 additional amino acids at their N-termini compared with AE1.[115]

a) Total protein 4.2 deficiency in homozygous band 3 gene mutations in animal models.

Total deficiency of protein 4.2 has also been reported in a complete lack of band 3 in red cell membranes; i.e., (1) in Japanese cattle due to a nonsense mutation of the band 3 gene,[63] (2) in knock-out mice by targeted disruption of the band 3 gene,[65] and (3) in the knock-out mice also by selectively targeted inactivation of the erythroid band 3 gene, in which kidney band 3 was not affected.[64]

A moderate anemia of autosomal incompletely dominant inheritance with marked microspherocyiosis[63] has been reported in Japanese cattle. In these

cattle, no band 3 was detected due to a nonsense mutation (CGA → TGA ; Arg → Stop) of the band 3 gene at the position corresponding to codon 646 in human red cell band 3 cDNA.[63] Immunoblotting analysis demonstrated very low content or nearly complete absence of protein 4.2 in red cell membranes of the proband.[63] Considerable decreases were also observed in other major red cell membrane components such as spectrin, actin, glyceraldehyde 3-phosphate dehydrogenase (band 6), and ankyrin (a reduction by at least 50% of normal subjects).[63] The probands also exhibited a marked distortion and disruption of the membrane skeletal network with tremendous instability.[63]

The affected cattle lacked kidney proteins, which are antigenically related to band 3, as in erythroid cells.[63] The proband red cells completely lacked rapid anion exchange as a function of band 3 protein ; i.e, the defective Cl^-/HCO_3^- exchange in these cells was uncompensated for and limited to a rather low level.[63]

Targeted disruption of the murine erythroid band 3 gene has resulted in spherocytosis and severe hemolytic anemia.[64] Erythroid band 3 gene was selectively inactivated but not the kidney band 3 gene. Red cells of homozygous mice were completely devoid of band 3 protein, whereas normal levels of band 3 protein were detected in the lysates of kidneys obtained from band $3^{-/-}$ mice.[64] Densitometric analysis of red cell membrane proteins indicated that the mutant ghosts contained a 75% of normal spectrin, significantly reduced ankyrin (40% of normal) and no detectable protein 4.2.[64] Normal amounts of protein 4.1 and actin were detected in the red cell membranes of homozygous mice.[64] The presence of a reduced but significant amount of ankyrin in band $3^{-/-}$ ghosts lends further support to the existence of band 3-independent sites for the attachment of ankyrin in the red cell membrane. Similarly, the presence of normal amounts of protein 4.1 in band $3^{-/-}$ ghosts indicates that the in vitro binding of protein 4.1 with band 3 may not occur in vivo. The concurrent loss of protein 4.2 in band $3^{-/-}$ red cells shows that the binding of protein 4.2 to the plasma membrane is exclusively determined by its interaction with band 3.[64] The red cell phenotype of the mice of this type is consistent with the results obtained from the cattle with the homozygous nonsense mutation of the band 3 gene.[63] The band $3^{-/-}$ red cells also contained adducin, dematin, P55, and glycophorin C.[116] In contrast, the band $3^{-/-}$ red cells are completely devoid of glycophorin A (GPA), although the polymerase chain reaction (PCR) confirmed the presence of GPA mRNA.[116]

The function of band 3 was also examined in the mice with targeted mutagenesis.[65] The mouse anion exchanger 1 (AE1) gene consists of 20 exons. An 1130 base pair segment between exons 9 and 11 was replaced with a neoR cassette. This segment encompasses the distal portion of the N-terminal cytoplasmic domain and the first membrane-spanning segment of the C-terminal domain. In the homozygous targeted mice, no AE1 transcript was detected in newborn reticulocyte or 14.5-day fetal liver RNA using a full-length AE1 cDNA.[65] No protein was detected using antibodies raised against either the cytoplasmic or membrane-spanning domains of AE1 in red cell membranes or

in whole cell lysates, confirming the absence of the normal AE1 gene product as well as the absence of any truncated AE1 polypeptides derived from the targeted gene.[65] $AE1^{-/-}$ red cell ghosts contained 84.7%±5.5%, 86.4%±5.4%, and 48.8% ±5.0% of wild type, steady state levels of α-spectrin, β-spectrin, and ankyrin, respectively.[65] The near normal spectrin content in AE1-deficient red cell membranes suggests the possibility of alternative membrane binding sites for spectrin or alternative mechanisms of assembling the membrane skeleton.[65] In $AE1^{+/-}$ red cells, normal amounts of α- and β-spectrin (94.8%±5.4% and 96.8%±4.2%, respectively) and ankyrin (119%±6.9%) but decreased levels of AE1 (82.3%±2.1%) were observed.[65]

In $AE1^{-/-}$ red cells, no protein 4.2 was detected, although these red cells retained 50% of the normal amount of ankyrin.[65] Therefore, it appears that AE1 contains the sole, high affinity binding site for protein 4.2.

b) Total protein 4.2 deficiency in human band 3 gene mutations.

Two kindred have been reported among patients with band 3 gene mutations in which protein 4.2 was totally or nearly completely missing in their red cells.

The first example is a Japanese family with four mutations on the band 3 gene[61]; i.e., two mutations of Memphis II polymorphism (K56E, AAG → GAG, and P854L, CCG → CTG) and, additionally, a mutation (G714IR, GGG → AGG) in one allele (allele Okinawa), and, *in trans* to allele Okinawa, a mutation (G130R, GGA → AGA) in another allele (allele Fukuoka), as shown in Table 3. The allele Fukuoka has been known to alter the binding of protein 4.2 to band 3.[60] The proband (the daughter) presented with a pronounced decrease of band 3 (49.8%±0.3% of normal), and showed an almost complete lack of protein 4.2 with only traces (less than 0.1% of normal) of 72, 68 and 66 kD fragments of protein 4.2.[60] (Table 2) Her mother showed a partial deficiency in band 3 (-25% on average) and a proportional reduction in protein 4.2.[60] Therefore, the mother was heterozygous for a novel allele of the EPB3 gene, allele Okinawa, and her daughter was a compound heterozygote of allele Okinawa and allele Fukuoka. Heterozygosity for allele Fukuoka has been documented in another individual who showed no clinical or hematological signs, and normal band 3 content.[60] It has been suggested that band 3 Okinawa binds virtually all the protein 4.2 in red cell precursors, band 3 Fukuoka being unable to do so, and that band 3 Okinawa cannot be incorporated into the membrane leading to degradation of the band 3 Okinawa protein complex.[60] In contrast, band 3 Fukuoka, free of bound protein 4.2, could then be incorporated normally into the lipid bilayer.[60] Thus, it has been speculated that protein 4.2 would not appear in the proband's red cell membranes.[60]

The second example of total protein 4.2 deficiency due to human band 3 gene mutation was found in a Portuguese baby with a missense mutation (band 3 Coimbra: V488M) in the homozygous state.[117,118] In a large Portuguese family, there was a couple whose members carried the mutation Coimbra in the heterozygous state. At the second pregnancy of this couple, homozygosity for mutation Coimbra was ascertained antenatally and the pregnancy was interrupted. At the third pregnancy, a severely anemic hydropic female baby in

the homozygous state was reanimated and kept alive with an intensive transfusional regimen. Cord blood smears disclosed dramatic erythroblastosis and poikilocytosis. Red cells with a tail-like elongation were a conspicuous feature. Band 3 and protein 4.2 were completely absent in red cell membranes. Metabolic acidosis and nephrocalcinosis were present. The total absence of band 3 in humans appears to be quite compatible with life as long as intensive transfusion support is provided. In the heterozygous state, the band 3 content, 4, 4'-diisothiocyano l, 2-diphenylethane-2, 2'-disulfonate (H_2DIDS) sites (μmol/L), and sulfate flux (nmol/10^8 red cells/10 min) were - 23% of normal on average, - 35% of normal, and - 34% of normal, respectively. Therefore, total protein 4.2 deficiency did occur in the homozygous state of the missense mutation of the band 3 gene.

2. Partial protein 4.2 deficiency

Partial deficiency of protein 4.2 is quite common in hereditary spherocytosis with band 3 mutations[1-3] under the two following situations.

i) Partial or total lack of one haploid set of mutated band 3.

The red cells lack one haploid set (a heterozygous state), partially or totally, of mutant band 3 (20~40% reduction of overall band 3), yielding mild to moderate hereditary spherocytosis with a dominant inheritance pattern. To date, numerous heterogenous mutations have been reported.[68,80,119-130] As a consequence, protein 4.2 is diminished in roughly the same proportions as band 3.

The first report to examine whether or not the product of the mutant allele is inserted into the membrane utilized one HS subject who was doubly heterozygous for the R760Q mutation and K56E (band $3^{Memphis}$) polymorphism of the human band 3 gene.[120] Only band $3^{Memphis}$ was detected in the red cell membrane, indicating that the protein product of the mutant (R760Q) band 3 allele was absent from the red cell membrane.

The same line of reports appeared in 166 families with autosomal dominant HS.[123] In these families. band 3 deficiency was invariably associated with mild autosomal dominant HS. They detected the first subset of band 3 gene mutations with seven nonsense and frameshift mutations that were all associated with absence of the mutant mRNA allele from reticulocyte RNA, implicating decreased production and/or stability of mutant mRNA as the cause of decreased band 3 synthesis.[123] The second subset included five substitutions of highly conserved amino acids and one in-frame deletion, which were associated with the presence of comparable levels ofnormal and mutant band 3 mRN.[123]

A French 18-year-old male demonstrated moderate HS with a 35% decrease in red cell band 3 content.[124] The underlying mutation was allele Lyon (R150X : CGA → TGA) with allele Genas, which was a G → A substitution at position 89 from the cap site in the 5'-untranslated region (89G → A). It has been shown that (1) the allele Genas (father) resulted in a 33% decrease in the amount of band 3 mRNA, (2) that the reduction caused by the allele Lyon (mother) was 42%, and (3) that the compound heterozygous state for both alleles (proband) resulted in a 58% decrease.[124]

It has also been shown that a mutant transcript is present in HS patients bearing missense mutations, whereas only the normal transcript is found in HS patients with frameshift mutations, in which the mean decrease in membrane band 3 content is significantly lower, leading to speculation that missense mutations may have some sort of dominant negative effect.[125]

Band 3 Foggia (311 del C; ACCCAC → ACCAC) and band 3 Napoli (447 ins T; TCT → TTCT) resulted in premature termination of translation, making one haploid set of band 3 mRNA unavailable,[126] as on band 3 Milano (Gln plus duplication of residues 478-499), which is probably not incorporated into the membrane.[128]

A nonsense mutation (Q330X) of the human red cell band 3 gene has been detected in HS.[129] This mutation was present in genomic DNA. In addition. a marked quantitative decrease in accumulation of the mutant band 3 RNA has been detected.[129]

In these situations, the extent of the decrease of protein 4.2 content was basically proportional to that of the band 3 content.

ii) Mutations in the cytoplasmic domain of band 3, which contains major binding sites for protein 4.2.

The decrement of the protein 4.2 content is unproportionally greater when mutations of the band 3 gene are present in its cytoplasmic domain, where the binding site(s) for protein 4.2 is located; e.g., band 3 Tuscaloosa (P327R: CCC → CGC),[58] band 3 Montefiore (E40K: GAG → AAG),[59] and band 3 Fukuoka (G130R: GGA → AGA).[60] The inheritance pattern is recessive for band 3 Montefiore and band 3 Fnkuoka. In these cases, protein 4.2 is sharply decreased due to the mutations on the band 3 gene.

A partial (29%±5%) deficiency of protein 4.2[58] was discovered in red cells of hereditary spherocytosis, in which one band 3 allele was normal but the other allele contained two mutations of the band 3 gene: (1) band 3 Memphis (K56E: AAG → GAG) and (2) band 3 Tuscaloosa (P327R: CCC → CGC). The predicted maximal binding capacity of the patient's inside-out vesicles (IOVs) for protein 4.2 was 33% lower than that of control IOVs (208±9 μg/mg compared with 312±1 μg/mg for control membranes).[58] The Kd for binding to patient membranes was also decreased nearly twofold ($2.4\pm0.2\times10^{-7}$ mol/L compared with $4.6\pm0.3\times10^{-7}$ mol/L for the control).[58]

A homozygous state for band $3^{\text{Montefiore}}$ (E232K: GAG → AAG) with a marked (88%) deficiency of protein 4.2 has been reported.[59] In the proband, the in vitro binding of protein 4.2 purified from control red cells to the proband's protein 4.2-stripped IOVs was decreased by 30% and 8% in two experiments, compared with normal controls.[59] However, the authors themselves had some reservation on this matter, because these small differences in binding were inconclusive and certainly could not explain the 88% protein 4.2 deficiency in the proposita's red cell membranes.[59] Levels of all other membrane proteins in this proband were normal except for band 6 (glyceraldehyde-3-phosphate dehydrogenase), which was 30% decreased.[59]

An extremely rare homozygous missense mutation of the band 3 gene (band 3 Fukuoka; Gl30R: GGA → AGA) showed substantial reduction (45.0%

of that of normal subjects) in addition to a minimal reduction (9.3%) of band 3 content.[60] Therefore, the extent of the decrement of protein 4.2 was unproportionally greater than that of band 3.[60] It is also noteworthy that, in addition to the 72 kD peptide (a wild type of protein 4.2), a trace amount of the 68 kD peptide was detected in the proband.[60] The extent of the rebinding of the proband's IOVs to the normal protein 4.2 was markedly reduced, compared with that of normal subjects. Scatchard plots indicated the average rebinding capacity in the proband was 207 μg of protein 4.2 per mg of vesicle proteins versus 295 μg in a normal subject. Therefore, the rebinding capacity of the mutated band 3 Fnkuoka to normal protein 4.2 appeared to be reduced to approximately 70% of the normal band 3.[60] Therefore, the unproportional reduction of protein 4.2 compared with that of band 3 was most likely due to the functional abnormality of the mutated band 3.[60]

Protein 4.2 variants : Protein 4.2 doublets

Two independent families with a doublet protein 4.2 in red cells, in which two protein 4.2 isoforms were present ; 72 kD as a wild type and 74 kD have been described.[5,6,110,131] The total amounts of protein 4.2, the sum of 72 and 74 kDs, were essentially normal.

The proband of the first family suffered from uncompensated hemolytic anemia with stomatocytosis.[5,6,131] This patient demonstrated a single band of 72 kD of protein 4.2 in the normal amount. In six out of ten family members, however, protein 4.2 consisted of two forms : 72 and 74 kDS in equal amounts.[131] These doublet cases (protein 4.2 doublet Kobe) also demonstrated stomatocytosis without any clinical symptoms. Four other family members showed only a single protein 4.2 (72 kD alone) with stomatocytosis, also without clinical symptoms.[131] In the red cells with the protein 4.2 doublet, sodium influx (1.5～1.9 mmols/ℓ RBC/hr ; normal : 1.29±0.14) and sodium efflux (3～7 mmols/ℓ RBC/hr ; normal : 2.4±0.5) were slightly enhanced.[131] Ektacytometry revealed normal rheological properties in the fresh intact red cells of these patients.[131]

The second family (two cases) also demonstrated a doublet with 72 and 74 kDs, but the distribution of these two isoforms was 70% for 72 kD and 30% for 74 kD (protein 4.2 doublet Nagano).[5,6,110] The sum of the 72 and 74 kD peptides was equivalent to the normal control, in which only 72 kD was present as a wild type. These patients showed overt hemolysis with marked reticulocytosis and increased MCHC.[110] The patient's red cell morphology demonstrated marked stomatocytosis and the presence of target cells. The two patients also demonstrated a marked increase in red cell membrane phosphatidylcholine, which would be responsible for target cells in the peripheral blood.[110] Full sequencing of the protein 4.2 gene by RT-PCR yielded only a normal gene size corresponding to its wild type (72 kD) with a heterozygous mutation of R488H,[110] which was confirmed in genomic DNA. The mutation was linked to the protein 4.2 doublet anomaly in the proband and his daughter, and not found in other family members, including a brother with normal 72 kD and membrane lipid anomalies.[110] The 74 kD of protein 4.2 appears to be derived from the protein 4.2 (72 kD) gene by the posttranslational modification, because the 90 nucleotide segment in exon 1 of

the protein 4.2 gene was skipped as usually observed in the wild type of protein 4.2 (72 kD) contrary to expectation, implying that the 74 kD was not produced by devoiding of the normal skipping of the 90 nucleotides in exon 1.[110] The intramembrane particles and skeletal network were nearly unaffected, as examined by electron microscopoy.[110] Concomitantly, red cell membrane lipid analysis in the proband revealed markedly increased free cholesterol (FC) (1681 $\mu g/10^{10}$ RBC ; normal : 1202 ± 103), and phosphatidylcholine (PC) (1063 $\mu g/10^{10}$ RBC ; normal : 733 ± 64).[110] The content of FC was 1384 $\mu g/10^{10}$ RBC in the brother and 1413 $\mu g/10^{10}$ RBC in the daughter, and that of PC was 943 $\mu g/10^{10}$ RBC in the brother and 865 in the daughter.[110] The composition of other phospholipids was essentially normal.[110] Other family members demonstrated no abnormalities of membrane proteins or membrane lipids.[110] Therefore, the membrane lipid abnormalities were proven to not be linked to the protein 4.2 doublet in the proband and his daughter, because his brother demonstrated the membrane lipid abnormalities in absence of the protein 4.2 doublet.[110]

Protein 4.2 isoforms have reportedly been observed in various animals.[132–134] In Oriental deer (*Cervus taiounus* and *Cervus nippon yesoensis*, *Heude*), 80% of a wild type consisted of a 78 kD peptide. As an isoform of protein 4.2, a 76 kD peptide was also present. In five out of 25 deer, a protein 4.2 doublet was detected, in which 78 and 76 kD peptides were present in equal amounts.

A null mutation ($4.2^{-/-}$) of protein 4.2 in mice

The red cell membrane protein 4.2 gene (Epb 4.2) has recently been targeted in embryonic stem (ES) cells to create a null mutation ($4.2^{-/-}$) in mice.[71] The mouse Epb 4.2 is ~22 kb in length and consists of 13 exons. A fragment extending from intron 3 to exon 8 was replaced with a neomycin-resistant cassette, removing exons 4 through 7 and part of exon 8. Homozygous null mutations were not distinguishable from normal littermates by phenotype at any age. Genotyping revealed the expected Mendelian frequency of homozygous null ($4.2^{-/-}$; 23%), heterozygous ($4.2^{+/-}$; 51%), and wild-type ($4.2^{+/+}$; 26%) offspring from heterozygous mating pairs.[71]

Protein 4.2 was not detected in $4.2^{-/-}$red cell ghosts on Coomassie blue-stained SDS-PAGE gels or by Western blotting. No protein 4.2 mRNA was detected by Northern blot analysis of $4.2^{-/-}$newborn reticulocyte RNA. The protein 4.2/spectrin ratios were 0.14 ± 0.01 in $4.2^{+/+}$ and 0.10 ± 0.01 in $4.2^{+/-}$, indicating that the content of protein 4.2 in red cells of heterozygotes ($4.2^{+/-}$) was decreased.[71]

Hematologically, $4.2^{-/-}$ mice had mild hereditary spherocytosis (HS).[71] Red cell counts and hematocrits were significantly reduced in $4.2^{-/-}$ mice ($9.7\pm0.2\times10^{12}$/L and 44.7 ± 0.9 versus $10.4\pm0.2\times10^{12}$/L and 51.8 ± 0.7 in $4.2^{+/+}$ mice). Reticulocyte percentages in $4.2^{-/-}$ mice were 5.5 ± 0.8% compared with 2.5 ± 0.1 in $4.2^{+/+}$ mice. The mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) were 46.3 ± 0.7 fL and 34.5 ± 0.5%, respectively, compared with 49.9 ± 0.8 fL and 31.9 ± 0.4% in $4.2^{+/+}$ mice. All hematological parameters were normal in $4.2^{+/-}$ mice. Red cell morphology demonstrated the presence of spherocytosis in $4.2^{-/-}$ mice, and both normal

biconcave red cells and intermediate, cup-shaped cells in $4.2^{+/-}$ mice. The deformability index was lower than normal in $4.2^{+/-}$ red cells and was further decreased in $4.2^{-/-}$ mice.

In protein chemistry, the band 3 content of $4.2^{-}/^{-}$ red cells appeared to be decreased on SDS-PAGE gels and Western blots.[71] The band 3/spectrin ratio was 1.00 ± 0.03 in $4.2^{-/-}$ mice and 1.19 ± 0.07 in $4.2^{+/+}$ mice. Net DIDS-sensitive sulfate influx was decreased to ~60% of wild-type in $4.2^{-/-}$ mice. In $4.2^{+/-}$ red cells, the band 3/spectrin ratio was also decreased (1.12 ± 0.04), and the net DIDS-sensitive sulfate influx was ~80% of normal. In $4.2^{-/-}$ red cells, normal amounts of spectrin, ankyrin, protein 4.1, p55, and glycophorin C were observed.

Ultrastructurally, a normal membrane skeleton has been reported to be assembled in $4.2^{-/-}$ red cells, despite the absence of protein 4.2 and partial band 3 deficiency.[71] However, the decreased number of intramembrane particles (IMPs) that remained were clustered in $4.2^{-/-}$ red cells.[71] It was speculated that this was owing to destabilization of the membrane due to a lack of horizontal lipid-protein interactions in those areas that were relatively devoid of integral membrane proteins within the bilayer.

In $4.2^{-/-}$ red cells, the Na^+ content was increased and the K^+ content was decreased.[71] The absolute K^+ loss exceeded the Na^+ gain, resulting in dehydration. No abnormalities in the Na^+ and K^+ contents were observed in $4.2^{+/-}$ red cells.[71] The maximal rates of the Na-K pump were identical among the three genotypes. There was a small but significant increase in the activity of the K-Cl cotransporter (Cl-dependent efflux),[135] significant increases in the activities of the bumetanide-sensitive Na-K-2Cl cotransporter and calcium-stimulated K^+ efflux (Gardos) channel, dramatic increase in the activity of the Na/H exchanger, a small increase in passive Na^+ permeability, and normal passive K^+ permeability in $4.2^{-/-}$ red cells.[71] The increased transport activities and passive Na permeability observed in $4.2^{-/-}$ red cells were due to increased sensitivity to cell shrinkage. It has been shown that increased Na-K-2Cl cotransporter and Na/H exchanger activities correlate with a net increase in phosphorylation. In $4.2^{-/-}$ red cells, cytosolic protein kinase C (PKC) was significantly decreased with decreased PKC-α and PKC-βI isoforms but normal PKC-βII.[71] Cytosolic protein kinase A (PKA) activity was increased in $4.2^{-/-}$ red cells.[71] Basal phosphorylation was increased and PMA-stimulated phosphorylation was reduced in $4.2^{-/-}$ red cell membranes, in which cytosolic casein kinase I (CKI) activity was normal with decreased cytosolic CKII.[71] The functional significance of these findings remains to be elucidated in the future. In addition. the contribution of partial band 3 deficiency to $4.2^{-/-}$ red cell cation transport is also unknown.

In nonerythroid expression of protein 4.2, protein 4.2 was present in normal platelets and absent from $4.2^{-/-}$ platelets, indicating that protein 4.2 in platelets was a product of the same gene (Epb 4.2) as was red cell protein 4.2.[71] A normal platelet count, however, was observed ($1{,}112\pm130\times10^3/\mu$L in $4.2^{-/-}$, $1{,}177\pm108$ in $42^{+/-}$).[71] Histological examination of brain spinal cord, heart, lung, liver, kidney, intestine, and muscle revealed no overt pathological change in $4.2^{-/-}$ mice at two or nine months of age.[71]

In summary, most of the observations in 4.2 knock-out mice are compatible and confirmatory with those observed previously in human patients with total deficiency of red cell protein 4.2.[5,6,31-33,67] Some findings, however, differ from those in human cases, especially in heterozygotes.[5,6,33,67] Human heterozygotes were totally silent in clinical hematology with normal red cell indices and normal reticulocyte counts.[5,6,33] In addition, no abnormalities were observed in red cell membrane proteins, even in protein 4.2 content and red cell cation content.[5,6,33] These minor discrepancies could be due to a species difference in some part and also to the artificial gene manipulation in the protein 4.2 knock-out mice. Conserving evaluation of the tremendous instability of skeletal network especially under a heated condition, which was observed in human protein 4.2 deficiency,[5,6,33,67] the authors did not perform these experiments in $4.2^{-/-}$ mice.[71]

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