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Selective Solubilization of a Protein Component of the Red Cell Membrane

Abstract. *Approximately 20 percent of the membrane-bound protein of erythrocyte ghosts can be solubilized and obtained free of other membrane components by dialysis against adenosine triphosphate and 2-mercaptoethanol. This protein forms one major band on polyacrylamide gels and a single boundary in free-boundary electrophoresis, and it undergoes polymerization in the presence of divalent cations to form coiled filaments visible by electron microscopy. Antibodies to this membrane protein react specifically with red blood cells or their membrane ghosts but do not react with serum, erythrocyte cytoplasm, or other blood cells. The functional role of this protein is unknown, but it appears to be involved in maintaining the structure of the red cell membrane. We suggest that this protein be called Spectrin since it is obtained from membrane ghosts.*

Although approximately one-half of the dry mass of most animal cell membranes consists of protein, very little is known about its chemical composition and physical properties. One of the major problems is the difficulty in separating proteins from the lipid and carbohydrate elements of membranes during solubilization without subjecting them to the denaturing effects of organic solvents or detergents.

It has been shown that trypsinized erythrocyte ghost membranes contain a protein or proteins capable of polymerizing into coiled filaments which are visible by electron microscopy (*1*). These filaments can be extracted in a soluble form from the digested membranes by dialysis against adenosine triphosphate (ATP) and reformed in vitro by incubating the extracted material with divalent cations. These findings suggested that part of the membrane protein was bound loosely to other membrane components which might be solubilized by extraction with ATP without treatment of the membranes with trypsin.

Membranes were prepared from guinea pig red blood cells by an osmotic lysis method (*2*). The washed membranes were extracted by dialysis against 0.3 m*M* ATP (*p*H 7.5) and 50 m*M* 2-mercaptoethanol at 4°C for 24 to 48 hours (*3*).

As a result of this treatment, the membranes break up into small fragments. Studies of thin sections by electron microscopy indicate that these fragments of membrane are made up of the usual three-layered structure, but that they differ from the original ghost membranes in two ways: (i) filamentous material normally present along the inner surface of intact membranes (*2*) is not attached to the fragments; and (ii) the edges of the fragments are "free" and do not form closed vesicles.

These fragments form a pellet after centrifugation at 78,000*g* for 2 hours, and the resulting supernatant contains approximately 20 percent of the original membrane-bound protein (*4*). This solubilized protein has a typical protein ultraviolet spectrum with a maximum absorbance at 282 mμ and contains no detectable lipid or carbohydrate moieties (*5*). The material forms one major band and one minor one on polyacrylamide gel electrophoresis in 8*M* urea (Fig. 1). A single boundary forms when the protein is run in free-boundary electrophoresis over a *p*H range of 6.5 to 9.8.

The extracted protein also forms coiled filaments similar in size and configuration to those in ghost membranes treated with trypsin (*1*). The filaments are produced by incubating the extracted protein with 0.5 m*M* ATP and 0.1 to 1.0 m*M* $MgCl_2$ or $CaCl_2$ at 37°C. They can be visualized directly by electron microscopy after negative staining with 2-percent phosphotungstic acid (PTA) (Fig. 2). The filaments measure approximately 40 to 60 Å in diameter and are of variable length.

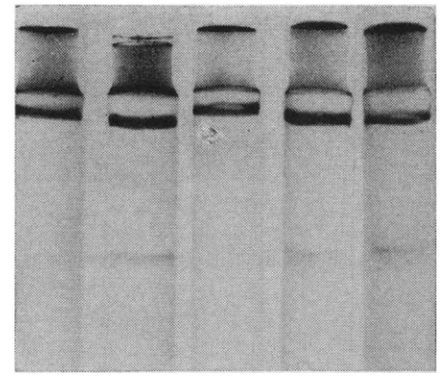

Fig. 1. Electrophoretic patterns of protein from red cell ghosts in 5-percent polyacrylamide gels containing 8*M* urea. The tubes represent protein extracted from five different red cell preparations. Gels were stained for protein with 0.5 percent Amido-Schwartz.

Antibodies to this protein were prepared by immunizing rabbits with the extracted material mixed with Freund's complete adjuvant. The antiserums obtained from these animals form a single precipitin zone with the extracted membrane protein after double diffusion in agar. No precipitin reaction occurs with either guinea pig serum or the red cell hemolyzate. Decomplemented antiserums agglutinate both intact red blood cells and their ghosts. Lysis of such antibody-treated cells occurs after the addition of serum containing active complement. Antiserums which agglu-

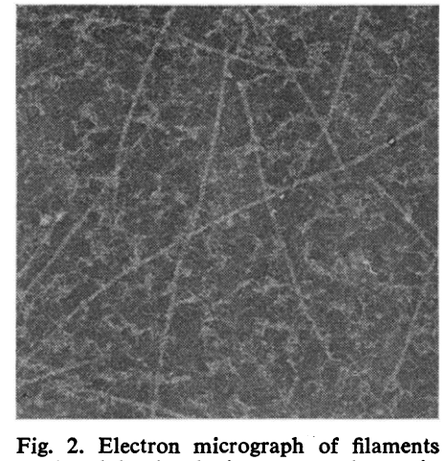

Fig. 2. Electron micrograph of filaments produced by incubating extracted protein with ATP (0.5 m*M*) and $MgCl_2$ (1.0 m*M*) at 37°C for 15 minutes. Preparation negatively stained with 2 percent PTA neutralized to *p*H 7.0 with KOH. The protein not organized in filaments appears as amorphous material in the background. We assume that this amorphous appearance is due to incomplete polymerization of the main component, because the extracted protein has this appearance when negatively stained without previous incubation with divalent cations (× 180,000).

tinate guinea pig erythrocytes do not agglutinate washed blood platelets from the same animals. Similarly, fluorescein-conjugated antiserums react strongly with intact erythrocytes and their ghosts but do not show any labeling with platelets or leukocytes.

Our studies show that approximately 20 percent of the protein bound to washed, erythrocyte ghost membranes can be readily solubilized and obtained free of other membrane elements simply by dialysis against ATP and 2-mercaptoethanol. This protein is different antigenically from the serum proteins and cytoplasmic proteins released from erythrocytes during hemolysis. This protein also has the remarkable property of polymerizing in the presence of divalent cations to form coiled filaments similar in the ultrastructure to the actin filaments of muscle. However, the physical, chemical, and antigenic properties of the erythrocyte membrane protein are quite different from those of muscle actin extracted from the same animals (*6*).

It is not yet clear where and in what form this protein is bound to the intact erythrocyte membrane. In that filamentous material is lost from the inner surface of the ghost membrane as a result of the extraction procedure used, this is probably a source of the soluble protein. However, the agglutination of intact red blood cells by antiserums prepared against the extracted protein suggests that antigenic material is also accessible on the outer surface of the membrane. Whether the same antigenic material is present on both the inner and outer surfaces of the membrane is unknown; it is possible that the antiserums contain antibodies to a minor protein component which are not detectable by the double-diffusion assay.

Antibodies directed against the extracted protein react only with red blood cells and their ghost membranes; other blood cells from the same animals are nonreactive. This specific reaction against erythrocyte membranes suggests either that they have a unique membrane protein or that this type of protein—if more widespread—is antigenically specific for different cell types. The distribution and cross reactivity of membrane proteins of other cell types remain to be determined.

The functional role of the protein in the intact erythrocyte membrane cannot yet be determined. Because the ghosts break up into small fragments under the conditions in which the protein is solubilized, it seems likely that this protein is somehow involved in maintaining the structure of the ghost membrane. Because this protein appears to be a new molecular species and is extractable from erythrocyte ghost membranes, we suggest that it be called Spectrin (Latin: derived from ghosts).

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3. Samples of washed membranes ranging from 0.5 to 1.0 g dry weight were dialyzed against 5 liters of a solution of ATP and mercaptoethanol for 24 hours at 4°C. The most recent preparations were subjected to a second 5-liter dialysis under the same conditions. This method was devised with the idea that this loosely bound protein component had solubility properties similar to those of muscle actin. This protein can also be solubilized from membranes treated with ethylenediaminetetraacetate in other media of low ionic strength, such as distilled water. It is not clear whether ATP and 2-mercaptoethanol stabilize the protein once it is free of the membrane.
4. Protein was measured by the Lowry procedure [O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)] with bovine serum albumin as the standard.
5. Samples of extracted protein were analyzed for sialic acid by the thiobarbituric acid method [L. Warren, *J. Biol. Chem.* **234**, 1971 (1959)]; neutral sugars, by paper chromatography; amino sugars, by amino acid analyzer; and sterols and phospholipids, by gas chromatography. We thank Drs. V. Ginsburg and E. Korn for help in these analyses.
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7. We thank Dr. G. E. Palade for criticism of the manuscript.

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Hemoglobin Hijiyama: A New Fast-Moving Hemoglobin in a Japanese Family

Abstract. *A variant of hemoglobin A, named Hb Hijiyama, found in two generations of a Japanese family living in Hiroshima, Japan, has a higher anodal electrophoretic mobility than hemoglobin A; a gain of two negative charges per molecule is indicated. Fingerprinting and amino acid analysis showed the biochemical anomaly to be in the beta chain at residue 120, where lysine is replaced by glutamic acid. In the heterozygote carriers of the abnormal hemoglobin there is no apparent association with clinical or hematologic abnormalities.*

During a systematic survey for hemoglobinopathies among visitors to the outpatient clinic of the Atomic Bomb Casualty Commission (*1*) in Hiroshima, Japan, between June 1963 and November 1965, 9262 blood samples were screened for abnormal hemoglobins by agar-gel electrophoresis (*2*). Two abnormal hemoglobins, both β-chain anomalies, were discovered. We now describe identification of the anomaly of one of these hemoglobins, which has been named Hb Hijiyama.

The index case was a 53-year-old Japanese housewife in good health; her red-blood-cell indices, morphology, and fragility were within normal limits. Of four children, only her eldest son, a 23-year-old university student in excellent health, had the abnormal hemoglobin; his red-cell indices and morphology were normal. The proband's parents were deceased. Blood samples from three paternal uncles and an aunt living in Hawaii were normal (*3*).

Agar-gel electrophoresis at *p*H 8.6 of hemolysates from the proband or her son produced two bands: one was in the position of Hb A; the other migrated more rapidly toward the anode. For estimation of the magnitude of the negative charge, Hb A and Hb Hofu ($\alpha_2\beta_2^{126\ Glu}$) (*4*) were compared with Hb Hijiyama on starch-gel electrophoresis (*5*). The distance between Hb A and Hb Hijiyama was approximately

Table 1. Comparison of amino acid contents of aminoethylated peptides from the abnormal spot of Hb Hijiyama with those of βTp-12B and βTp-13 of Hb A. The values found for Hb Hijiyama were obtained by automatic amino acid analysis (see text); those expected for Hb A are given by Jones (*10*) and Braunitzer *et al.* (*14*). Abbreviations: Lys, lysine; His, histidine; Thr, threonine; Glu, glutamine or glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine.

Amino acid	Residues (No.)		
	Hb Hijiyama abnormal peptide		Hb A βTp-12B + βTp-13, expected
	Found	Integral	
Lys	0.87	1	2
His	2.03	2	2
Thr	1.04	1	1
Glu	3.58	4	3
Pro	1.95	2	2
Gly	1.19	1	1
Ala	2.80	3	3
Val	1.77	2	2
Leu	0.96	1	1
Tyr	.86	1	1
Phe	2.27	2	2