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PERIPHERAL PROTEINS OF HUMAN ERYTHROCYTES

J.E. Deas, L.T. Lee, and C. Howe

Departments of Tropical Medicine and Medical Parasitology
and Microbiology

Louisiana State University Medical Center
New Orleans, Louisiana 70112

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SUMMARY

Water soluble, nonglycosylated proteins have been extracted from human erythrocyte membranes by two different methods and characterized immunochemically and by PAGE. The spectrin peripheral protein complex (PAGE bands 1 + 2) has been equated with two antigens of intermediate mobility in immunoelectrophoretic analysis of crude spectrin developed with antiserum to bands 1 + 2 purified by elution from gels. Nonspectrin proteins, including catalase, remain in close association with isolated membranes, and display solubility properties similar to those of spectrin. Along with spectrin, they may also function in the intact cell as peripheral proteins.

INTRODUCTION

Peripheral proteins of the erythrocyte membrane interact electrostatically with cytoplasmic termini of transmembranal integral proteins, the external ends of which bear various receptor groupings at the cell surface. The peripheral proteins are therefore by definition those which can be separated from isolated membranes in aqueous suspension by manipulation of pH and ionic strength. Fibrillar structures, with characteristic electron microscopic appearance, have been described in the protein fraction extractible from erythrocytes incubated in aqueous media of low ionic strength in the absence of divalent cations (1,2). These fibrils, which form when the protein mixture is incubated with Mg++, were originally thought to represent a single class of proteins and were given the name spectrin. Spectrin was found to be associated exclusively with the inner aspect of the membrane (3), and has been identified as peptides 1 + 2 in the currently accepted profile of normal human erythrocyte membranes (4).

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We report here the isolation of the spectrin complex (bands 1 + 2) and show that it is antigenically distinct from other membrane proteins, some of which may have functions analogous to those of spectrin.

MATERIALS AND METHODS

Single donor units of blood were obtained from the New Orleans Blood Bank prior to expiration dates, and were washed thoroughly to remove plasma, platelets and leukocytes. Erythrocytes were then lysed in 20 mOsM phosphate buffer, pH 7.4, containing 10^{-4} M EDTA. After centrifugation at 22,000 g, sedimented membranes were processed further (5), as shown in Figure 1. The resulting products were soluble ghost protein (SGP) (6) and crude spectrin (2), from both of which single proteins were subsequently isolated. Antisera were prepared in rabbits. Each animal received three weekly footpad or intramuscular injections of 0.4-0.5ml protein (1.2 mg/ml) in complete Freund's adjuvant, and was bled out 10-14 days after the final injection. Electrophoretic and immunoelectrophoretic analyses in agarose gel were done as previously described (6). Two dimensional immunoelectrophoresis was done according to methods described by Laurell (7) and by Ollier and Hartmann (8). Polyacrylamide gel electrophoresis (PAGE) with or without SDS was carried out according to Maizel (9) using 7.5% gels in 0.05 M Tris glycine buffer, pH 8.6 at 2 mA per gel. Gels were fixed and stained with Coomassie brilliant blue (CBB) according to Fairbanks, et al. (4). Preparative PAGE was performed for 4 - 5 hr. at 20 mA per gel in 7.5% gels, in 0.05 M Tris glycine buffer, pH 8.6. An aliquot of 1.5 - 2.0 mg crude spectrin was applied to each gel. Bands 1 + 2, representing the spectrin complex, were located in the slab gel by comparison with a parallel strip cut from the center of the gel and stained. Proteins were eluted into Tris glycine buffer without detergent. Gel separation and elution were repeated at least once in order to achieve maximal purification of spectrin. Residual unbound SDS was removed from eluates by filtration through Triton X-100 agarose (10). Protein was determined by the method of Lowry (11).

RESULTS AND DISCUSSION

We determined by electron microscopy of negatively stained preparations that both crude spectrin and SGP contained the characteristic fibrillar structures originally described as spectrin (1). In the separation, on Sephadex G200, of hemoglobin from other proteins in crude spectrin, catalase comigrated with the spectrin complex. On SDS PAGE, catalase dissociated into subunits which separated from bands 1 + 2. The latter, as finally eluted from preparative SDS gels of crude spectrin (Fig. 2a), were free of other proteins, as shown by analytical PAGE analysis (Fig. 2b), and contained no components stainable with periodic acid Schiff reagents (PAS). The purity of the eluted product was further established by the absence of antibody to nonspectrin proteins in antisera made to PAGE-purified spectrin

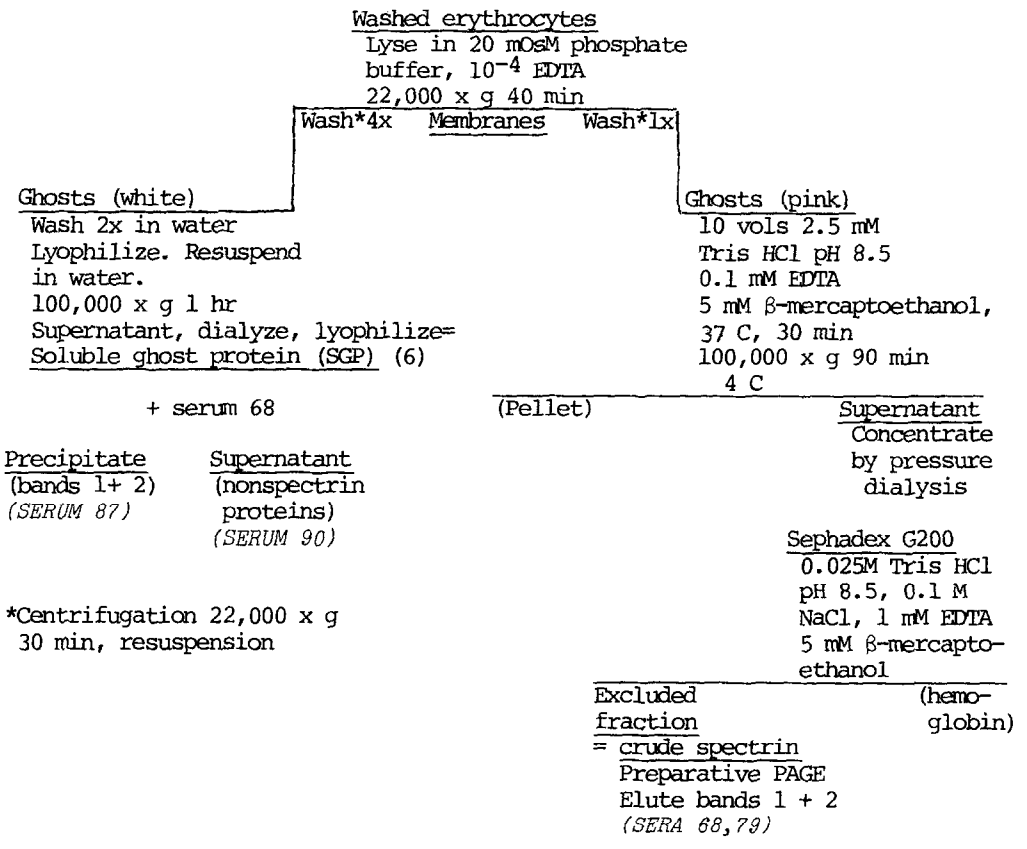

Figure 1. Preparation of erythrocyte fractions

(Figs. 4,5). Specific precipitates obtained from SGP by reaction with antibody to PAGE-purified bands 1 + 2(Fig. 1, serum 68) contained only the spectrin complex, as judged in immunoelectrophoretic analysis of crude spectrin with the same antiserum (Fig. 5a).

In two dimensional immunoelectrophoretic analysis (Fig. 4), developed with antisera to PAGE-purified spectrin (Fig. 1, serum 68) and immune-precipitated spectrin (Fig. 1, serum 87), crude spectrin gave a pattern consisting of two overlapping bands. These corresponded with two parallel precipitin arcs revealed in immunoelectrophoretic analysis of crude spectrin developed with antiserum to PAGE-purified bands 1 + 2 (Fig. 5a, serum 79, and with PAGE bands 1 + 2.

The consistent presence in spectrin of two antigens of similar mobility

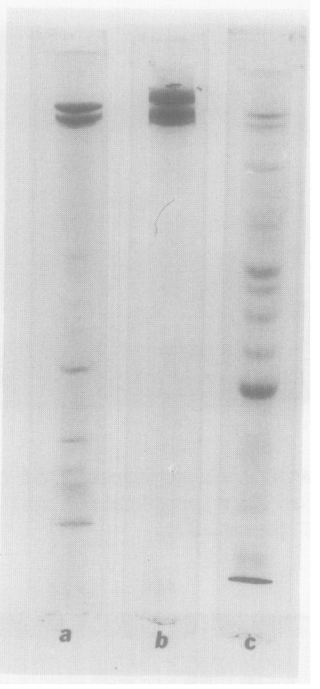

Figure 2. a) Crude spectrin, 25 µg protein. b) Spectrin (bands 1 + 2) eluted from preparative SDS gel, 20 µg protein. c) Soluble ghost protein (SGP), 40 µg protein.

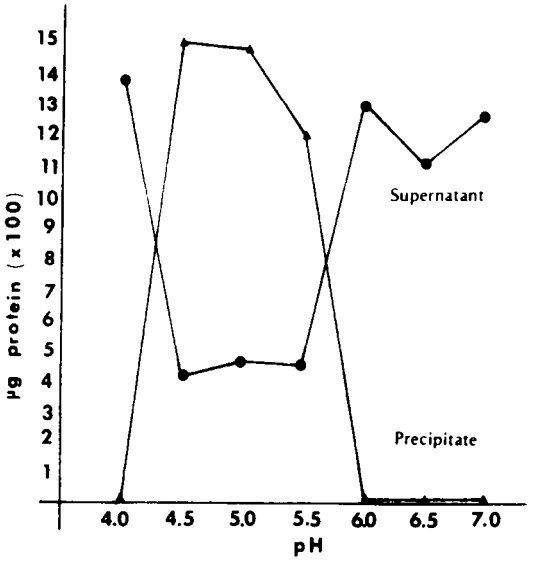

Figure 3. Solubility curve of soluble ghost protein (SGP). Equal aliquots of protein were dialyzed against buffers at pH indicated, centrifuged. Precipitates made up to original volume in buffer pH 7.4 and supernatants measured for total protein by Lowry (11).

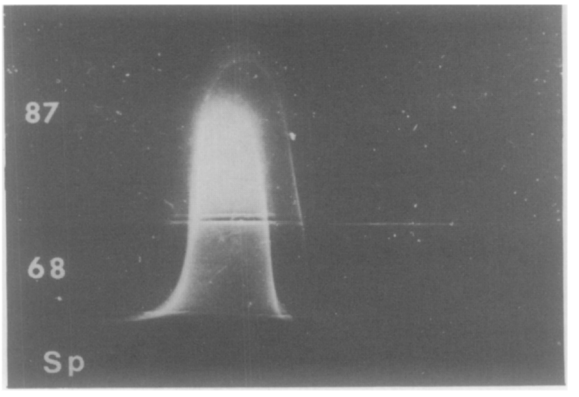

Figure 4. Two dimensional immunoelectrophoresis of crude spectrin against antisera to bands 1 + 2 eluted from preparative gel (serum 68) and to immune-precipitated spectrin (serum 87).

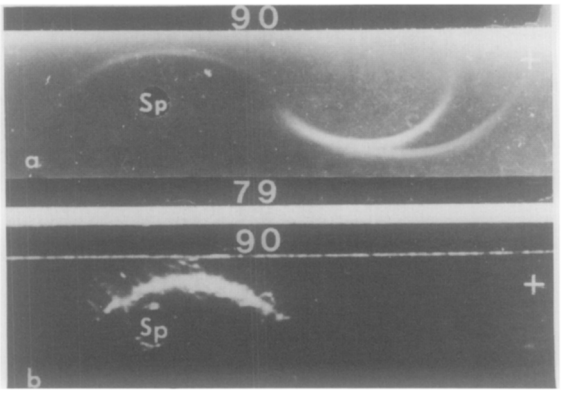

Figure 5. a) Immunoelectrophoresis of crude spectrin against antisera to nonspectrin proteins of SGP (serum 90) and to spectrin purified by immunoprecipitation (serum 79). b) Direct catalase reaction in immune precipitate of catalase with serum 90.

(Figs. 4,5) is compatible with the doublet configuration seen on PAGE (Fig. 1b). The constant relationship of these bands to one another indicates that the two proteins are of similar size and charge, although they appear to be antigenically distinct from one another. Related observations have been made by Dunn, et al. (12) who reported each band to be a heterogeneous mixture of at least 8 peptides, some of which occurred in both bands.

The internal localization of spectrin and nonspectrin proteins was substantiated by the failure of corresponding antisera (Nos. 68,79,90) to agglutinate intact erythrocytes. In contrast, antisera to integral proteins, portions of which are exposed at the cell surface, agglutinated erythrocytes of all three blood groups to high titer. The specificity of these latter agglutinins is independent of ABO determinants, and is directed toward species specific integral protein determinants common to erythrocytes of all groups (6). We have further determined that the spectrin complex is antigenically distinct from the integral proteins (results not shown), chiefly glycophorin (13).

Crude spectrin and SGP contained essentially the same array of polypeptides, but in different proportions. In crude spectrin (Fig. 2a), bands 1 + 2 were the major components, clearly separable from all the rest by elution from preparative gels (Fig. 2b). In SGP, bands 1 + 2 were less prominent than in crude spectrin, but the reverse was true of at least 5 smaller components which were present in higher relative concentration than in crude spectrin. For the preparation of spectrin (Fig. 1) ghosts which had been derived by hypotonic hemolysis and washed only once in hypotonic buffer are extracted in alkaline buffer with EDTA and β-mercaptoethanol. This procedure liberates peripheral proteins from the membranes, which are then removed by ultracentrifugation. Hemoglobin and lower molecular weight proteins are then taken out on Sephadex G200, bands 1 + 2 being the main components found in the void volume proteins constituting crude spectrin. In preparing SGP, on the other hand, ghosts are washed 6 X in hypotonic buffer, a condition which promotes further detachment of peripheral proteins from the membranes. However, the resulting ghosts retain the biconcave shape and the full complement of lipids characteristic of intact erythrocytes in isotonic medium, as well as a constant protein profile definable immunologically (6). However, a group of nonglycosylated proteins can still be separated simply by rehydration and ultracentrifugation of

lyophilized ghosts (Fig. 1, SGP). Even after this final aqueous extraction, the integral proteins (2,4,6) remain bound with lipid and are therefore insoluble in water.

It remains to be determined which of the soluble proteins besides bands 1 + 2 are functional peripheral proteins and which represent predominantly cytoplasmic constituents artifically occluded to the membranes as a result of cytolysis and other preparative manipulations. As noted, 5 or 6 components of molecular weight lower than bands 1 + 2 are found in both crude spectrin and SGP (Figs. 2a,c), the former being enriched in bands 1 + 2. All of the nonspectrin proteins in SGP may not function as peripheral proteins in the intact cell. However, an alternative to this should be considered.

Peripheral erythrocyte proteins, including spectrin (14), have been reported to precipitate at pH 4.5 - 5.5, as we have also shown (Fig. 3). Even after removal of spectrin by immunoprecipitation, the nonspectrin proteins were still maximally precipitated at pH 4.5. This finding implicates them, along with spectrin, in the aggregation of membrane-associated particles (MAP) which occurs at low pH and which has been ascribed to precipitation of residual spectrin adherent to membranes (15, 16). Maximal flocculation of erythrocyte membranes derived by hypotonic lysis also occurs at about pH 5.5 (17). This pH-dependent effect is doubtless dependent upon, if not the result of these perturbations mediated by spectrin and perhaps other soluble ghost proteins which have similar solubility properties.

Among the nonspectrin proteins in SGP, catalase was prominently identified (Fig. 5b). Catalase is a hemoprotein which contains ferriprotoporphyrin. It has a molecular weight of 240,000 in subunits of 60,000, and an isoelectric point of 5.6 (18). This enzyme is an important component of the erythrocyte cytosol, although it has been identified in the membrane profile as a part of band 4.5 in the nomenclature of Fairbanks, et al. (4), the amount remaining bound being augmented in the presence of calcium or in

metabolically stressed cells (19). Our results indicate that a significant amount of the enzyme remained with the membranes regardless of the procedure used in their preparation, and that loss of spectrin paralleled loss of catalase during repeated washing of isolated membranes in hypotonic buffer as in the preparation of SGP (Fig. 1), in which both, however, were still present. All of the foregoing considerations prompt the suggestion that catalase may also be a peripheral protein with functions heretofore ascribed to spectrin alone.

ATPase activity has been identified in band 2 of crude spectrin (20). On further purification of spectrin, ATPase activity disappeared, probably due to loss of some necessary co-factor, perhaps analogous to troponin or tropomyosin of skeletal muscle. It is conceivable that some other "contaminant" may serve this co-factor function, possibly one of the nonspectrin components which we have found to be precipitable at pH 4.5.

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