SPECTRIN IN PRIMITIVE ERYTHROCYTES

Jennifer C. PINDER, Joanne PHETHEAN* and W. B. GRATZER
Medical Research Council Cell Biophysics Unit, King's College, Drury Lane, London, WC2B 5RL, England

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1. Introduction

The high-molecular weight protein, spectrin, comprises about one quarter of the total protein complement of the human erythrocyte membrane. It is a complex of two types of chain with molecular weights of about 2.2 and 2.4 × 10^5, which are present in equimolar proportions [1]. The function of this protein is still uncertain, but there is considerable evidence [2] to link it to a contractile role, essential for the maintaince of cell shape and flexibility. The question then arises whether spectrin or a close counterpart exerts a similar control over the properties of other cell types. High-molecular weight proteins have certainly been found in many other cells [3,4], and electrophoretic components similar in appearance to the spectrin doublet have been observed (and tentatively identified with spectrin) in the periacrosomal material of *Thyone* sperm [5]. On the other hand, Hiller and Weber [6] have reported that spectrin is absent from a range of tissue-culture cells.

To date therefore the only unequivocal identification of spectrin is confined to mammalian erythrocytes. By gel electrophoresis in the presence of sodium dodecyl sulphate (SDS), the total erythrocyte membrane protein pattern is strongly conserved between the mammalian species so far examined [7–9], and the spectrin doublet is indistinguishably present in all of them, including the camel [10], the cells of which are not discoid in shape. The immunological similarity of spectrins from several species has also been reported [8].

In attempting a further assessment of the degree of ubiquity of spectrin, we have examined the membranes of the large and complex erythrocytes of the most primitive animal to enclose its respiratory pigment in cells, viz the marine invertebrate, *Terebella lapidaria*. The cell membranes cross-react with rabbit anti-spectrin and the site of the cross-reactivity is a single chain of molecular weight 270 000. The results of this study establish the prevalence of spectrin in erythroid cells.

* Sandwich Course Student, Hatfield Polytechnic, 1975–1976

2. Materials and methods

2.1. *Erythrocytes and membranes*

Erythrocytes from the vertebrate species were washed four times with an isotonic saline solution, viz 0.15 M sodium chloride, 5 mM phosphate buffer, pH 8.0 (PBS), and used directly. *Terebella lapidaria* were obtained from the Marine Biology Laboratory, Plymouth, U.K. Vascular blood was drawn by insertion of a finely drawn Pasteur pipette, and transferred immediately to cold isotonic buffer, containing anticoagulant (0.15 M sodium chloride, 5 mM phosphate, pH 7.5, with 8.5% sucrose and 200 units/ml heparin). The cells were washed as before, and lysed in 10 volumes of 5 mM phosphate, pH 7.5. To isolate the plasma membrane free of organelles, isopycnic sedimentation in a sucrose gradient was performed: 1 ml of the cell lysate was applied to the top of a preformed stepped sucrose gradient (0.5–2.0 M in 0.5 M steps in 5 mM phosphate buffer, pH 7.5) in a 5 ml tube; after centrifugation at 40 000 rev./min in a swing-out rotor for 2½ h, the membrane fraction could be observed as a refractile zone in the vicinity of the

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1.0–1.5 M sucrose interface. This fraction was collected and examined in the microscope under phase contrast.

2.2. *Electrophoresis*

Samples (50 μl washed packed cells) were mixed with 50 μl 10% sodium dodecyl sulphate (SDS), then diluted to 500 μl with 0.125 M Tris, pH 6.8, 1% β-mercaptoethanol, 5% sucrose, 0.001% Bromophenol Blue, and heated to 100°C for 5 min; 5–50-μl samples were applied to slab polyacrylamide gels, 20 × 20 × 0.2 cm, and subjected to electrophoresis in a discontinuous Tris–glycine buffer system, containing 0.1% sodium dodecyl sulphate [11]. The gels were stained with 0.05% Coomassie Brilliant Blue R in methanol:acetic acid:water (5:1:5 v/v) and destained in 10% acetic acid.

2.3. *Immunological procedures*

2.3.1. Preparation of antispectrin antiserum

10 ml pre-immune serum was taken from each of three New Zealand white rabbits, checked for absence of cross-reactivity with spectrin on Ouchterlony plates and used as a control. The rabbits were immunised with human spectrin, prepared according to Marchesi [12] and purified by chromatography on a Sephadex G-200 column, as described earlier [13]. The following schedule was followed: 650 μg protein in Freund's complete adjuvant were injected intramuscularly in the upper hing leg, followed after 5 weeks by a further 150 μg, in Freund's incomplete adjuvant, subcutaneously in multiple sites in the neck. The rabbits were bled from the ear vein, and the serum checked for antispectrin antibodies 7 days after booster immunisations of 100–200 μg alum-precipitated spectrin in PBS, pH 7.4, at 3 weeks intervals.

2.3.2. Immunofluorescence

Erythrocytes washed in PBS, pH 7.0, were smeared onto glass slides, and allowed to dry in air. Prior to staining, slides were immersed in cold acetone for 5 min, air-dried again and washed for 5 min in PBS. Cells were stained with antisera (5:1 dilution with PBS) or preimmune serum (at equivalent dilution) for 20 min. After a 20-min wash in the buffer, the cells were stained with fluorescein-conjugated goat anti-rabbit immunoglobulin (Miles-Seravac), 10 times diluted. The slides were washed in buffer for 20 min, and examined in a Zeiss Photomicroscope, using epifluorescence optics and a ×40 oil-immersion objective.

2.3.3. Iodination of erythrocyte proteins

Terebella erythrocytes solubilised in SDS were diluted 50-fold with 0.1 M borate buffer, pH 8.5. To 500 μl of this solution were added 0.2 μg (approx. 500 μCi) solid ^{125}I-*N*-succinimidyl 3-(4-hydroxyphenyl) propionate [14] in a stoppered tube, which was left in ice for 15 min. Iodination of amino groups was terminated by addition of 500 μl 0.2 M glycine in borate buffer, pH 8.5, and reaction products were removed by passage through a 5 cm column of Sephadex G-25. Protein was dialysed against PBS, at pH 7.0, and stored frozen at −70°C.

2.3.4. Antibody precipitation of antispectrin cross-reactive *Terebella* protein

200 μl samples of iodinated protein was incubated at 4°C for 24 h with 15 μl antispectrin antiserum, or the pre-immune rabbit serum as control. Rabbit antibodies were precipitated by the addition of 150 μl goat anti-rabbit immunoglobulin, followed by incubation at 4°C for 24 h. Antibody complexes were collected by centrifugation, and washed and dissolved for electrophoresis in the SDS-containing sample buffer. Gels were exposed for autoradiography using Kodak Kodirex X-ray film for 7 days.

2.3.5. Fluorescence depolarisation

Polarisation of emission from 1-anilino-8-napthalene sulphonate (ANS) (purified by two re-crystallisations from water) was measured in a Perkin-Elmer Hitachi MPF-3L spectrofluorimeter, with Polacoat polarisers in the excitation and emission beams. Standard corrections were applied for intrinsic polarisation of the incident radiation [15]. The reagent at a final concentration of 1 mM was introduced into the ghost suspension [16], and measurements were performed after the signal had become constant. The temperature was maintained at 20°C.

3. Results

3.1. *Vertebrate erythrocytes*

The erythrocyte ghosts of mouse, chicken, frog and

newt all gave rise to immunofluorescence when stained by the indirect technique with antiserum to human spectrin. Hardly any effect could be detected in the fluorescence microscope when cells were stained with pre-immune rabbit serum at an equivalent dilution. SDS-gel electrophoresis of the total ghost protein showed that, as expected [7,9], the mammalian species (mouse) gave rise to two components in the high-molecular weight region, indistinguishable from human spectrin. The chicken showed, in accordance with Chan [17] a triplet, with two bands resembling those of human spectrin and a third of unknown identity. The newt gave a two-component pattern, displaced, however, to slightly higher molecular weight than human, whereas the frog showed one well-defined and some minor zones in the same region.

Terebella erythrocytes are large cells (20–30 μm in diameter), and apparently spherical. SDS-gel electrophoresis of the total erythrocyte protein shows the presence of several high-molecular weight zones, and examination of the membrane fractions from the isopycnic sucrose gradients (fig.1) shows that these are associated with the membranes. Indirect immunofluorescent staining, using antispectrin antiserum (fig.1), caused strong surface fluorescence of the cells, indicating that spectrin is present and apparently distributed through the membrane. Preimmune rabbit serum controls showed a low level of background staining.

The *Terebella* erythrocyte protein was labelled with

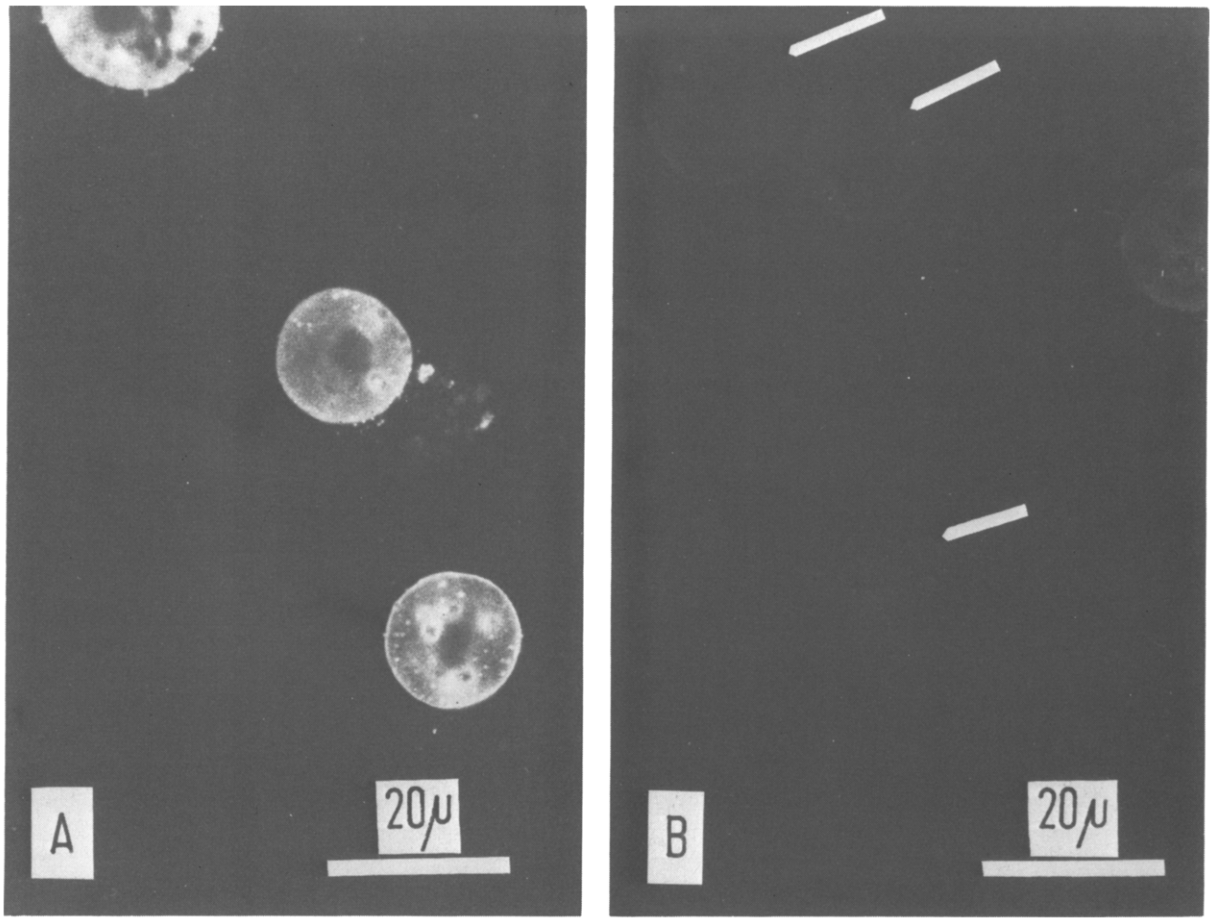

Fig.1. Indirect immunofluorescence of *Terebella lapidaria* erythrocytes, reacted with anti-human spectrin-specific immunoglobulin fraction (A), and treated with identical concentration of pre-immune immunoglobulin (B), (for details see text).

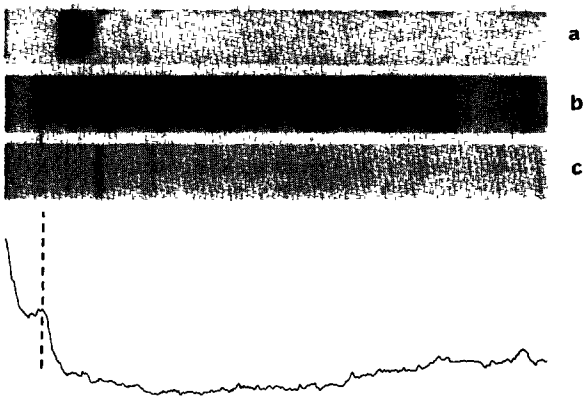

Fig.2. Gel electrophoresis in SDS of redissolved precipitin pellet of *Terebella lapidaria* membrane protein. (a) Human spectrin; (b) total *Terebella* erythrocyte protein; (c) *Terebella* erythrocyte membrane protein, all stained with Coomassie Brilliant Blue. The densitometer trace is of an autoradiogram of the dissolved radioiodinelabelled precipitin pellet. The high-molecular weight protein peak corresponds to the stained zone indicated.

radio-iodine; SDS-gel electrophoresis of the redissolved precipitin pellet produced by reaction of goat anti-rabbit antibodies with the incubated mixture of anti-spectrin and the radio-labelled erythrocyte protein, showed the presence of a single cross-reacting species (fig.2). Its electrophoretic mobility in the SDS system corresponds to a molecular weight of 2.7×10^5. Comparison of the stained gel with that of total *Terebella* erythrocyte protein makes it possible to identify one of the bands in the membrane extracts as the cross-reacting protein, which may be operationally described as a spectrin. It is only one of several components apparent in the high-molecular weight range. Because the fluidity of the erythrocyte membrane is evidently regulated in some sense by the spectrin network [18], we have carried out measurements of fluorescence depolarisation of ANS in the ghost membranes [16]. Because of the many imponderable factors in the quantitative interpretation of polarisation in terms of viscosity of the medium, and its dependence on ANS concentration and other variables [19], we confine ourselves to a comparison of the polarisations obtained under identical conditions for ANS in human and *Terebella* cells. The polarisations observed with both systems were rather similar, that of the *Terebella* membrane being consistently slightly higher (ratio 1:18).

4. Discussion

Our results indicate that proteins operationally identifiable as spectrins are present in vertebrate erythrocytes. This is consistent with its presumed involvement in the control of the rheological properties of the membrane, on which the cell depends in a circulatory system, necessitating its passage through capillaries. In this light the presence of spectrin in the terebellid worm is less to be expected. Its identification is based on the following criteria: the protein resides in the membrane, it has a high subunit molecular weight, and it cross-reacts strongly with anti-human spectrin antibodies.

Spectrin is thus evidently present in all evolutionary stages of erythroid cell development. The cells of *Terebella* which coexist with a haemolymph respiratory system, are complex, and serve other functions than oxygen transport, for they contain glycogen granules, mitochondria and other organelles. Despite the very different circumstances in which they are designed to function, they not only possess spectrin, but their plasma membrane is evidently quite similar in terms of microviscosity to those of man.

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