Human Erythrocyte Myosin: Identification and Purification

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ABSTRACT   Human erythrocytes contain an M_r 200,000 polypeptide that cross-reacts specifically with affinity-purified antibodies to the M_r 200,000 heavy chain of human platelet myosin. Immunofluorescence staining of formaldehyde-fixed erythrocytes demonstrated that the immunoreactive myosin polypeptide is present in all cells and is localized in a punctate pattern throughout the cell. Between 20–40% of the immunoreactive myosin polypeptide remained associated with the membranes after hemolysis and preparation of ghosts, suggesting that it may be bound to the membrane cytoskeleton as well as being present in the cytosol. The immunoreactive myosin polypeptide was purified from the hemolysate to ~85% purity by DEAE-cellulose chromatography followed by gel filtration on Sephacryl S-400. The purified protein is an authentic vertebrate myosin with two globular heads at the end of a rod-like tail ~150-nm long, as visualized by rotary shadowing of individual molecules, and with two light chains (M_r 25,000 and 19,500) in association with the M_r 200,000 heavy chain. Peptide maps of the M_r 200,000 heavy chains of erythrocyte and platelet myosin were seen to be nearly identical, but the proteins are distinct since the platelet myosin light chains migrate differently on SDS gels (M_r 20,000 and 17,000). The erythrocyte myosin formed bipolar filaments 0.3–0.4-μm long at physiological salt concentrations and exhibited a characteristic pattern of myosin ATPase activities with EDTA, Ca^{++}, and Mg^{++}-ATPase activities in 0.5 M KCl of 0.38, 0.48, and <0.01 μmol/min per mg. The Mg^{++}-ATPase activity of erythrocyte myosin in 0.06 M KCl (<0.01 μmol/min per mg) was not stimulated by the addition of rabbit muscle F-actin. The erythrocyte myosin was present in about 6,000 copies per cell, in a ratio of 80 actin monomers for every myosin molecule, which is an amount comparable to actin/myosin ratios in other nonmuscle cells. The erythrocyte myosin could function together with tropomyosin on the erythrocyte membrane (Fowler, V. M., and V. Bennett, 1984, J. Biol. Chem., 259:5978–5989) in an actomyosin contractile apparatus responsible for ATP-dependent changes in erythrocyte shape.

Underlying the plasma membrane of eucaryotic cells is a cytoskeletal actin filament network that is believed to play a structural role in determining cell architecture as well as a dynamic role in generating membrane movements. The molecular organization of this membrane cytoskeleton is best understood in the human erythrocyte, a cell with a unique biconcave disk shape, remarkable deformability properties, and no intracellular membranes or organelles. The available evidence indicates that the erythrocyte membrane cytoskeleton is constructed of many short actin filaments (~12–20 monomers long) (26, 35) that are cross-linked into an anastomosing network in the plane of the membrane by long, flexible spectrin molecules in association with an M_r ~80,000

helper protein, band 4.1. The entire cytoskeletal ensemble is attached to the cytoplasmic surface of the membrane via the specific association of spectrin with ankyrin, an M_r ~210,000 protein that is itself tightly bound to the cytoplasmic domain of band 3, the anion channel, and major integral membrane protein (for recent reviews, see references 5, 7). The recent isolation of spectrin- and ankyrin-like proteins from nonerythrocyte cells and tissues and their localization on the plasma membrane (4, 10) encourage the view that the organization of the erythrocyte membrane cytoskeleton could indeed be representative of the plasma membrane cytoskeleton of nucleated cells, at least in certain regions. However, as currently depicted, this model is an essentially static one and

does not account for the plasma membrane movements of nucleated cells (e.g., membrane ruffling, filopodial extension and retraction, and endo- and exocytosis), nor for dynamic ATP-dependent discocyte-echinocyte shape transformations of erythrocytes (22, 24, 34, 37).

We describe here the identification and purification of myosin from human erythrocytes. The erythrocyte myosin was found to be present with respect to the erythrocyte actin in a ratio of about 80 actin monomers to 1 myosin molecule, an amount comparable to actin/myosin ratios in other non-muscle cells. In addition, we have recently identified a non-muscle from of tropomyosin on the erythrocyte membrane that is present in sufficient quantities to almost completely coat all of the short actin filaments in the membrane cytoskeleton (14). This suggests that the erythrocyte myosin is not simply a relic from a previous developmental stage of the cell and supports the hypothesis that a membrane-associated actomyosin contractile apparatus could be responsible for ATP-dependent changes in erythrocyte shape and deformability.

MATERIALS AND METHODS

Production and Purification of Antibodies: Myosin was purified from outdated human platelets as described by Pollard et al. (28) and was stored at 4°C as a slurry in 60% saturated ammonium sulfate. Three New Zealand white rabbits were injected subcutaneously at multiple sites on the back and sides with 100–150 μg each of native myosin in complete Freund's adjuvant, followed by booster injections with antigen in incomplete Freund's adjuvant after 3 wk, and then at 1–2-mo intervals thereafter. Titer was monitored by the immunoblot method (see below) and was high after the third injection. Immune serum was diluted with 1 vol of 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, 1 mM NaN₃, 0.2% (vol/vol) Triton X-100, heated to 60°C in the presence of 200 μg/ml phenylmethylsulfonyl fluoride to minimize protease activity, and stored at $-20°C$. Antibody against platelet myosin was isolated by affinity chromatography with myosin coupled to cyanogen bromide-activated Sepharose (C1) 4B (Pharmacia Fine Chemicals, Piscataway, NJ) (0.7 mg of myosin/ml of agarose). 10–20 ml of immune serum was applied to a 3.5-ml column, the column was washed, and antibody was eluted as described previously (14). Peak fractions (based on A₂₈₀) were pooled, concentrated three- to fourfold by dialysis into 150 mM NaCl, 10 mM sodium phosphate, 1 mM Na EDTA, 0.02% NaN₃, pH 7.5, 50% glycerol at 4°C, and stored at $-20°C$. Yields of purified antibody ranged from 0.03 to 0.10 mg (based on $E_{280}^{1\%} = 14$) per milliliter of diluted serum, depending on the rabbit. Although the antibodies were isolated by affinity chromatography with native platelet myosin, they were specific for the heavy chain and did not label the light chains in immunoblotting assays. Ig was isolated from preimmune serum by affinity chromatography on Protein A-Sepharose (14). Affinity-purified antibodies to human erythrocyte spectrin were a gift from Dr. Peter C. Agre (Department of Medicine, The Johns Hopkins University School of Medicine).

Preparation of Hemolysate, Membranes, and Purified Proteins: Erythrocytes were isolated from freshly drawn human blood anticoagulated with acid/citrate/dextrose as described (6). Cells were lysed in 10–15 vol of ice-cold 7.5 mM sodium phosphate, 1 mM EDTA, 2 mM dithiothreitol (DTT),[1] 20 μg/ml of phenylmethylsulfonyl fluoride, pH 7.5, and the membranes were pelleted by centrifugation as described (6); this supernatant is referred to as the hemolysate. Membranes were prepared by three more washes in the lysis buffer (6). Ankyrin and spectrin were purified from membranes as described (6), and the M_r 200,000 immunoreactive myosin polypeptide from the hemolysate as described in the text and the legend to Fig. 3. Protein concentrations for the purified proteins were determined spectrophotometrically from the absorbance at 280 nm (after correction for light scattering at 320 nm) based on an $E_{280}^{1\%}$ value of 10 for spectrin and ankyrin, and 5.9 for myosin (28).

Electrophoresis and Immunoblotting Procedures: Electrophoresis was performed on 5–15% acrylamide linear gradient SDS gels in the presence of 4 M urea, with a 5% stacking gel containing 2 M urea, and with sample preparation and molecular weight standards as described previously (14). Electrophoretic transfer of polypeptides from the SDS gels to nitrocellulose paper was performed as described previously (14) except that gels were trans-

[1] *Abbreviation used in this paper:* DTT, dithiothreitol.

ferred for 6 h at 500–700 mA current at 15°C. Nitrocellulose gel transfers were labeled overnight (12–18 h) at 4°C with 2–4 μg/ml of affinity-purified antibodies to human platelet myosin, and processed for antibody detection with ¹²⁵I-Protein A (2 × 10⁶ cpm/ml) as described (14).

We quantitated the amount of the M_r 200,000 immunoreactive myosin polypeptide in a sample by staining the protein on the nitrocellulose transfers with 0.2% ponceau S in 3% trichloroacetic acid (10 min at room temperature followed by two washes in distilled water), cutting out the appropriate region of the nitrocellulose, and counting the ¹²⁵I-Protein A-labeled band in the gamma counter. The actual micrograms of the immunoreactive myosin polypeptide were computed from a standard curve prepared from immunoblots of known amounts of purified erythrocyte myosin that were electrophoresed on SDS gels and transferred to nitrocellulose in parallel with the unknowns. The standard curve was linear from ~0.02 to at least 0.25 μg of protein, and duplicate samples were reproducible ± 5%. The concentration of the purified erythrocyte myosin for the standard curve was based on the A₂₈₀, assuming $E_{280}^{1\%} = 5.9$ (28), and corrected for the purity of the preparation (~85%; see Results) by multiplying by a factor of 1.2.

Immunofluorescence of Erythrocytes: Erythrocytes (isolated as described above) were fixed in suspension (10% [vol/vol]) in 3% (wt/vol) paraformaldehyde in PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.5) for 15 min at room temperature, and then allowed to settle onto poly-L-lysine-coated coverslips (No. 2) for an additional 5 min at room temperature. Coverslips were washed three times in PBS, incubated 30 min at room temperature in 50 mM NH₄Cl in PBS, and then washed three times in PBS. Cells on coverslips were permeabilized by a 30-s incubation in acetone at $-20°C$, transferred immediately to PBS containing 0.2% gelatin (PBS/gelatin), and then washed two times in PBS/gelatin. Coverslips were placed over 50-μl drops of affinity-purified antibodies or preimmune Ig (10 μg/ml in PBS/gelatin) and incubated for 30 min at room temperature in a humid chamber. After four washes in PBS/gelatin they were incubated for 20 min at room temperature with tetramethyl rhodamine isothiocyanate goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN) (1/400 dilution in PBS/gelatin). Following two washes in PBS/gelatin and two washes in PBS, coverslips were mounted on slides in 50% glycerol, 50% PBS and examined in a Zeiss microscope equipped with epifluorescence optics. Micrographs were taken with Kodak Tri-X pan film, ASA 800.

Antimyosin staining of erythrocytes was only obtained with the formaldehyde fixation, acetone postfixation/permeabilization protocol described above. Formaldehyde fixation followed by permeabilization with 0.1% Triton X-100 resulted in extraction of the phase-dense cytoplasmic contents of the cells and completely eliminated the antimyosin staining. Also, no specific staining was visible with acetone treatment alone in the absence of formaldehyde fixation. The antispectrin staining was identical regardless of the fixation/permeabilization protocol.

Electron Microscopy: For rotary shadowing, purified erythrocyte myosin in 0.5 M KCl was dialyzed at a concentration of 30 μg/ml against 100 mM ammonium formate, 30% (vol/vol) glycerol, pH 7.0, sprayed onto freshly cleaved mica, dried under vacuum at room temperature, and rotary shadowed at an angle of 1–10° with platinum followed by carbon (36). For negative staining, purified myosin in 0.5 M KCl was dialyzed at a concentration of 200 μg/ml against 50 mM KCl, 2 mM MgCl₂, 10 mM PIPES, pH 7.0, 4°C, to form filaments. After dilution and application to Formvar- and carbon-coated grids, the myosin was negatively stained with 1% uranyl acetate. Rotary shadowed and negatively stained samples were photographed using a Zeiss microscope operating at 80 kV.

RESULTS

Identification of Myosin in Human Erythrocytes

Previous attempts to identify myosin in human erythrocytes have been frustrated by the similarity in molecular weight of the myosin heavy chain (M_r 200,000) to the major membrane-associated cytoskeletal proteins spectrin (M_r of band 2 ~220,000) and ankyrin (M_r ~210,000), and by high levels of ATP hydrolysis resulting from membrane-associated ion pumps and coupled kinase-phosphatase activities in whole-cell lysates, membranes, and membrane extracts. To circumvent these problems, we prepared antibodies to platelet myosin to use as a specific probe in assaying for the presence of myosin in erythrocytes. Fig. 1 shows that affinity-purified antibodies to the M_r 200,000 heavy chain of human platelet myosin cross-reacted with an M_r 200,000 polypeptide in

A Coomassie Blue   **B** Immunoblot

anti-platelet
myosin   pre-immune Ig

1 2   3 4 5 6   1 2 3 4 5 6   1 2 3 4 5 6

260—
220—

94—

60—
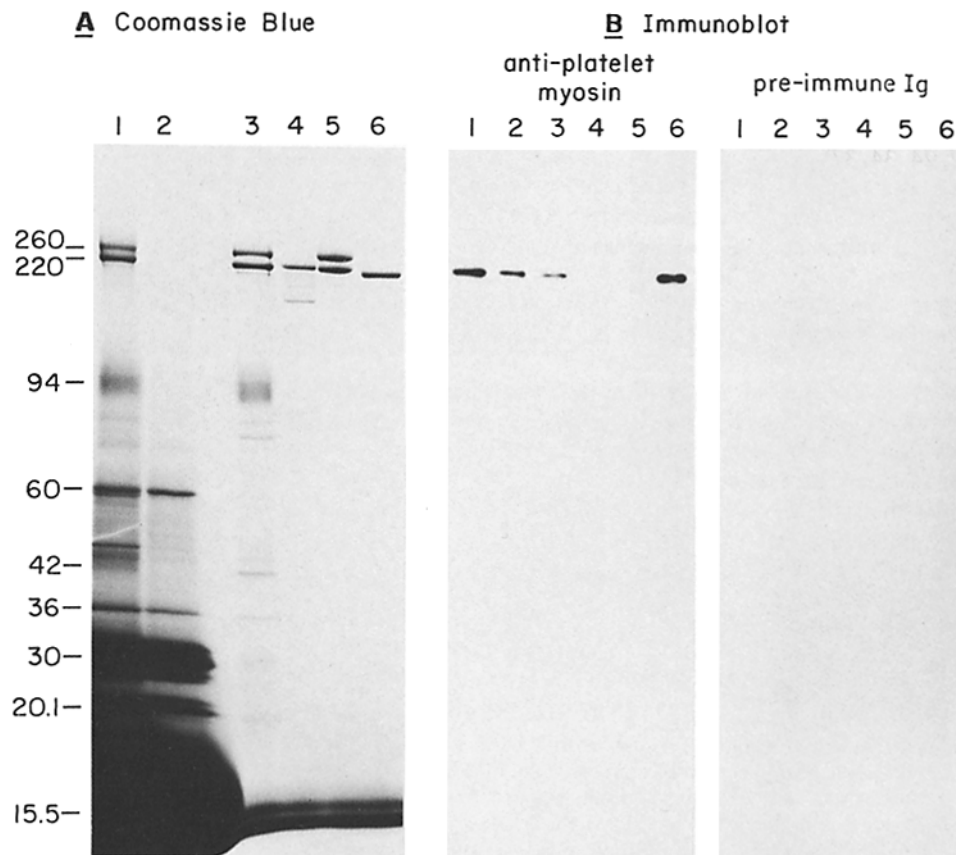
42—
36—
30—
20.1—

15.5—

FIGURE 1   Identification of an M_r 200,000 immunoreactive myosin polypeptide in human erythrocytes. (Lane *1*) Erythrocytes; (lane *2*) cytosol; (lane *3*) membranes; (lane *4*) erythrocyte ankyrin (1.5 μg); (lane *5*) erythrocyte spectrin (2 μg); (lane 6) platelet myosin. (Panel *A*, 1.6 μg; panel *B*, 0.08 μg). Erythrocytes were lysed in 15 vol of lysis buffer and membranes were prepared as described in Materials and Methods. Aliquots of the lysed erythrocytes, the hemolysate (cytosol), and the washed membranes resuspended to the initial lysis volume were added directly to SDS gel electrophoresis sample buffer (14) and heated to 80°C for 5 min. 80-μl samples of each were electrophoresed on 5–15% acrylamide linear gradient SDS gels in the presence of 4 M urea, and either (*A*) stained with Coomassie Blue or (*B*) electrophoretically transferred to nitrocellulose paper as described in Materials and Methods. The nitrocellulose strips were incubated with 2 μg/ml of affinity-purified antibodies to human platelet myosin, or preimmune Ig, followed by incubation with ^{125}I-labeled protein A. Immunoreactive bands were detected by autoradiography after exposure of the film for 1 h.

human erythrocytes. Preimmune Ig did not label either the purified platelet myosin (Fig. 1, lane 6) or the cross-reactive polypeptide in the erythrocytes (Fig. 1, lanes *1–3*). The immunoreactive polypeptide was not band 2 of spectrin or ankyrin (M_r equivalent to band 2 of spectrin on these gels), since neither purified ankyrin (Fig. 1, lane *4*) nor purified spectrin (Fig. 1, lane *5*) were labeled by the antimyosin antibodies. These results suggest that the immunoreactive M_r 200,000 polypeptide could be the M_r 200,000 heavy chain of an erythrocyte myosin-homologue.

To determine whether this immunoreactive myosin polypeptide was localized on the membrane or in the cytosol, we lysed erythrocytes and compared immunoblots of whole cells, hemolysates, and membranes (Fig. 1, lanes *1–3*). A true cytoplasmic component would be expected to be present in the hemolysate in the same proportion as in the whole cells, whereas a tightly bound membrane component would be expected to be associated exclusively with the washed membranes. Fig. 1 shows that only ~30–40% of the immunoreactive myosin polypeptide was released into the supernatant during hemolysis of erythrocytes in 7.5 mM sodium phosphate, pH 7.5 (Fig. 1, lane 2), while an additional 30–40% was washed off the membranes during the preparation of

ghosts (Fig. 1, lane *3*).[2] This intermediate fractionation behavior might be expected from a component that is loosely bound to the membrane as well as being present in the cytosol. However, a variable proportion of the putative myosin homologue may also be tightly associated with the membranes since ~20–30% of the immunoreactive myosin polypeptide remained associated with the membranes even in well-washed, white ghosts (Fig. 1, lane *3*), and inclusion of physiological concentrations of magnesium (2 mM) in the lysis and washing buffers resulted in about a twofold increase in this amount (data not shown).

Immunofluorescence staining of formaldehyde-fixed erythrocytes with the affinity-purified antibodies to myosin demonstrated that the immunoreactive myosin polypeptide was present in all cells (Fig. 2*a*). The putative erythrocyte myosin-homologue was thus not derived from reticulocytes or from contaminating platelets or neutrophils in the cell preparations. No staining with preimmune Ig was observed under these conditions (Fig. 2*c*). The antimyosin staining was only observed when the fixed cells were permeabilized with acetone

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[2] Estimated by quantitative immunoblotting procedures as described in Materials and Methods.
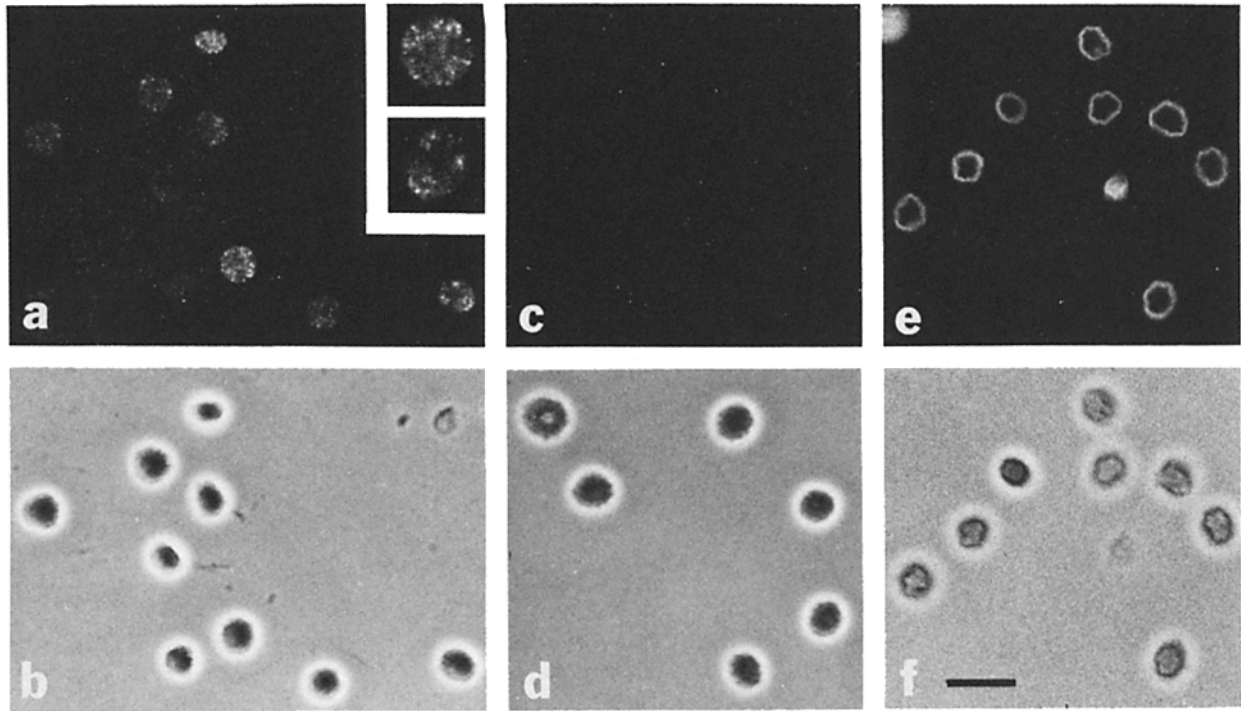
FIGURE 2 Immunofluorescence staining of human erythrocytes with antiplatelet myosin (a), preimmune Ig (c), and antispectrin (e). b, d, and f are phase-contrast micrographs corresponding to a, c, and e. Erythrocytes were fixed with 3% paraformaldehyde followed by acetone at −20°C and incubated with the various rabbit Ig samples at 10 μg/ml, followed by rhodamine-conjugated goat anti-rabbit IgG, as described in Materials and Methods. The antispectrin staining in e is actually 50–100-fold brighter than the antimyosin staining in a (estimated by the exposure times used to photograph the cells and to print the negatives), but the micrographs are printed so as to be on the same scale for comparison. (a–f) Bar, 10 μm. × 9,000. (Inset) × 18,000.

before incubation with the antibody, indicating that the immunoreactive myosin polypeptide was in the interior of the cell and not adsorbed adventitiously to the cell surface. The antimyosin staining was variable in intensity and was distributed in a granular or punctate pattern in each cell (Fig. 2a, see inset). The small size of the cells and the relatively dim staining made it difficult to determine whether the punctate antimyosin staining was localized in the cytosol and/or associated with the membrane in the fixed cells. The punctate staining pattern was probably not artifactually induced by the fixation/permeabilization protocol because staining with affinity-purified antibodies to spectrin produced a uniform rim-staining pattern (Fig. 2e), which is consistent with the established location of spectrin on the cytoplasmic surface of the membrane (5, 23, 39).

Purification and Partial Characterization of Erythrocyte Myosin

To evaluate the possibility that the immunoreactive myosin polypeptide is the M_r 200,000 heavy chain of an erythrocyte myosin-homologue, we purified the polypeptide from the hemolysate and compared its physical and functional properties with those established for authentic vertebrate myosins (19, 21, 29). The immunoreactive myosin polypeptide was separated from the enormous amount of hemoglobin, as well as numerous other cytosolic proteins in the hemolysate, by DEAE-cellulose chromatography in the presence of 20 mM sodium pyrophosphate, pH 7.5 (Fig. 3A, compare lanes 1 and 2). This procedure also served to concentrate the M_r 200,000 immunoreactive myosin polypeptide by about 25-fold with respect to its original concentration in the hemolysate (<1

μg/ml).[2] The M_r 200,000 polypeptide was then concentrated an additional 10-fold by precipitation with ammonium sulfate at 60% saturation (Fig. 3A, lane 3) and separated from most of the low molecular weight polypeptides by gel filtration in 0.5 M KCl on Sephacryl S-400 (Fig. 3A, lane 4). When more protein was loaded on the gels, two low molecular weight polypeptides (M_r 25,000 and 19,500) were seen to be associated with the purified M_r 200,000 polypeptide (Fig. 3B, lane 1). These polypeptides were present in a molar ratio of 1.09 M_r 25,000:0.90 M_r 19,500:1.0 M_r 200,000 polypeptide,[3] and thus presumably represent the two light chains that would be expected to be associated with the M_r 200,000 heavy chain of an erythrocyte myosin molecule. Together, these three polypeptides accounted for ~85% of the Coomassie Blue-staining material in the purified preparation. In the representative experiment shown in Fig. 3, ~0.7 mg of protein was obtained from two units of blood, which represents a recovery of ~18% of the protein present as the M_r 200,000 immunoreactive myosin polypeptide in the hemolysate.[2]

Structurally, the purified erythrocyte myosin is a typical vertebrate myosin, with two heads and a long rod-like tail ~150 nm long (21, 29, 36), as visualized by low angle rotary shadowing of individual molecules (Fig. 4, left column). The double-headed appearance of the individual molecules, together with the roughly equimolar stoichiometry of the M_r 200,000, 25,000, and 19,500 polypeptides (1:1.09:0.90, see above), suggests that the native molecules are dimers of two heavy chains, each with two associated light chains, as are other myosins. In addition, peptide maps of the M_r 200,000

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[3] Determined by quantitative elution of dye from the Coomassie Blue-stained protein bands as described by Fenner et al. (12).

A PURIFICATION OF ERYTHROCYTE MYOSIN

Coomassie Blue

Immunoblot

$M_r \times 10^{-3}$

B COMPARISON OF ERYTHROCYTE MYOSIN (I) AND PLATELET MYOSIN (2)
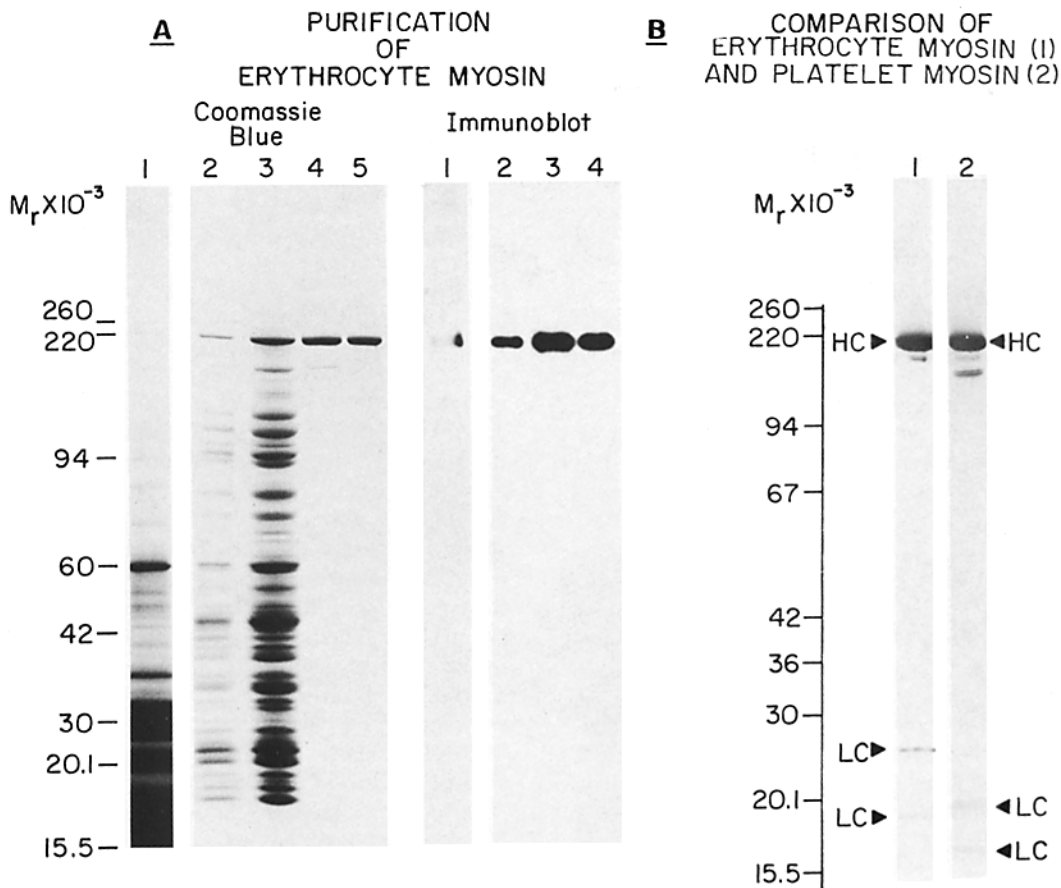
$M_r \times 10^{-3}$

FIGURE 3 (A) Purification of human erythrocyte myosin. Erythrocytes were isolated from two units of whole blood as described by Bennett (6) and lysed in 12 vol of ice-cold 7.5 mM sodium phosphate, 1 mM EDTA, 2 mM DTT, 20 µg/ml phenylmethylsulfonyl fluoride (pH 7.5), and the membranes were removed by centrifugation for 45 min at 17,000 g (2°C). The hemolysate (3,300 ml) was adsorbed batchwise to DEAE-cellulose equilibrated with 20 mM sodium pyrophosphate, 1 mM EDTA, 2 mM DTT (pH 7.5) for 1 h at 4°C with stirring, and unadsorbed protein (mainly hemoglobin) was removed by several cycles of settling and decanting of the DEAE in this buffer. The DEAE was poured into a column (2.5 × 18 cm), washed with 5 column volumes of 50 mM NaCl, 20 mM sodium pyrophosphate, 1 mM EDTA, 2 mM DTT (pH 7.5), and then eluted with 7 column volumes of a 50-250 mM NaCl linear gradient in the same buffer, collecting 10-ml fractions. Fractions containing the immunoreactive myosin polypeptide (assayed by immunoblotting procedures) were pooled (150 ml), and the protein was precipitated by addition of 1.5 vol of ice-cold, saturated ammonium sulfate and then collected by centrifugation for 20 min at 17,000 g. The precipitate was resuspended to a 13-ml final vol in 0.5 M KCl, 10 mM Tris, 1 mM EDTA, 1 mM DTT (pH 7.5), and centrifuged for 30 min at 100,000 g. The supernatant was applied to a Sephacryl S-400 column (2.5 × 85 cm) equilibrated with the same buffer and run at 20 ml/h, collecting 5-ml fractions. Fractions containing the M_r 200,000 polypeptide eluted at ~1.5 V_0 and were pooled (40 ml) and concentrated 5-10-fold to 0.1-0.2 mg/ml by dialysis against solid sucrose. The purified protein was stored on ice after dialysis against 0.5 M KCl, 10 mM 3-(N-morpholino) propane sulfonic acid, 1 mM EDTA, 1 mM DTT (pH 7.0) and was stable for at least 3 wk. Samples of hemolysate (80 µl) [lane 1], the DEAE pool (40 µl) [lane 2], the ammonium sulfate-concentrated material (20 µl) [lane 3], and the purified M_r 200,000 polypeptide (2 µg) [lane 4] were electrophoresed on 5-15% acrylamide linear gradient SDS gels containing 4 M urea and either stained with Coomassie Blue (left) or transferred to nitrocellulose and labeled with 4 µg/ml platelet myosin and ^{125}I-labeled protein A (right) as described in Materials and Methods. One tenth as much protein was loaded for the immunoblots, and the immunoreactive bands were detected after exposure of the autoradiograms for only 30 min. Lane 5 is 2 µg of purified human platelet myosin for comparison. (B) Comparison of 6 µg each of purified human erythrocyte myosin (lane 1) and human platelet myosin (lane 2) on 7.5-15% acrylamide linear gradient SDS gels in the absence of urea (14). HC, heavy chains; (LC), light chains.

heavy chain of the purified erythrocyte myosin and of platelet myosin were found to be nearly identical (Fig. 5), indicating a high degree of structural homology between the two proteins. However, these proteins are distinct in that the molecular weights determined for the platelet myosin light chains on 7.5-15% acrylamide linear gradient SDS gels (M_r 20,000 and 17,000; Fig. 3B, lane 2) were different from those determined for the erythrocyte myosin light chains (M_r 25,000 and 19,500; Fig. 3B, lane 1).

Dialysis of the purified erythrocyte myosin into low salt led

to formation of typical bipolar myosin filaments (0.3-0.4 µm long) with the heads at each end and a central bare zone in the middle (Fig. 4, middle and right column). Occasionally, possible intermediate stages in the formation of these filaments were observed in the rotary-shadowed specimens. For example, two molecules are associated via their tails in the bottom left panel of Fig. 4, and the head end of an individual myosin molecule is splayed out from the bipolar filament while the tail region remains attached in the filaments depicted in the bottom two panels of the middle column in Fig.

4. The looser association of the molecules in the rotary-shadowed specimens as compared with the negatively stained specimens may be due to the somewhat different dialysis conditions used for the sample preparations (0.1 M ammonium formate, pH 7.5, versus 50 mM KCl, 2 mM MgCl$_2$, pH 7.0), or it may be an artifact of the respective electron microscopic techniques.

The purified erythrocyte myosin exhibited a characteristic pattern of myosin ATPase activities, with a Ca^{++}-activated, Mg^{++}-inhibited ATPase activity at both high and low salt, and activation by high concentrations of KCl but not NaCl in the absence of divalent cations (2 mM EDTA) (Table I). The Ca^{+2}-ATPase activity in 0.5 M KCl of the erythrocyte myosin was higher than the EDTA-ATPase activity measured in 0.5 M KCl (0.48 and 0.38 μmol/min per mg, respectively). This is the reverse of the relative Ca^{++} and EDTA-ATPase activities of platelet myosin measured under similar condi-

tions (0.35 and 0.50 μmol/min per mg, respectively, see reference 28). The addition of rabbit skeletal muscle F-actin (1 mg/ml) to purified erythrocyte myosin had no effect on the low Mg^{++}-ATPase activity of erythrocyte myosin that was measured in 60 mM KCl, 2 mM MgCl$_2$, pH 7.0 (<0.01 μmol/min per mg).

Determination of the Number of Copies of Myosin per Cell

The purified erythrocyte myosin was used as a standard in a quantitative immunoblotting assay to determine the amount of myosin in erythrocytes (Materials and Methods). As shown in Table II, the amount of the M_r 200,000 heavy chain of erythrocyte myosin detected was directly proportional to the microliters of cell equivalents electrophoresed and transferred to nitrocellulose, which demonstrates that the assay is operating in both antimyosin antibody and ^{125}I-protein A excess. Additionally, this also shows that the co-migrating spectrin (band 2) and ankyrin polypeptides are not interfering with the transfer or antibody labeling of the erythrocyte myosin heavy chain. The molar ratio of the M_r 200,000 polypeptide to spectrin was calculated with respect to the micrograms of band 1 of spectrin in each sample because there are no polypeptides co-migrating with band 1 of spectrin on these SDS gels of erythrocytes. The average value for the ratio of spectrin to myosin calculated from the values in Table I was 32.1 spectrin dimers/myosin molecule (dimer of two heavy chains). Since there are 200,000 molecules of spectrin dimer per cell (5), the number of molecules of myosin per cell would be 6,240. This is a ratio of about 80 actin monomers for every myosin molecule, assuming there are 500,000 copies of actin per cell (5, 26).

DISCUSSION

The possibility that erythrocytes contain myosin has been discussed since 1960 when Nakao et al. (22) first observed the reversible ATP-dependent discocyte-echinocyte shape transformations of human erythrocytes. The ability of isolated membranes (ghosts) to undergo similar ATP-dependent shape transformations (24, 34, 37), as well as ATP-dependent endocytosis (25, 31), led numerous investigators to hypothesize
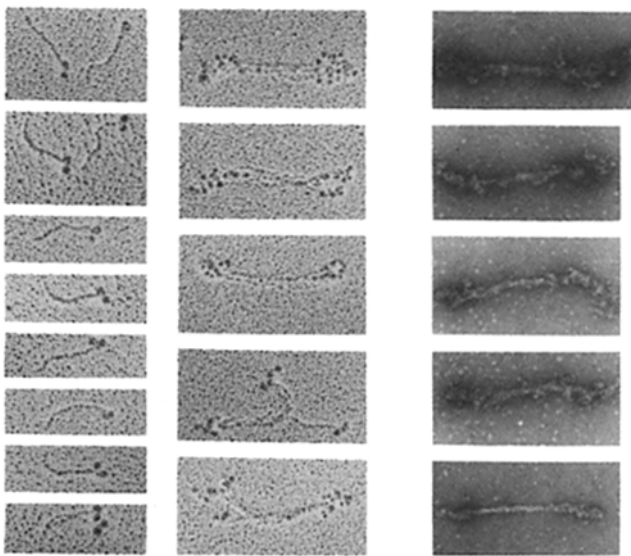

Figure 4 Electron micrographs of low angle, rotary-shadowed individual human erthyrocyte myosin molecules (left) and myosin filaments (middle), and of negatively stained myosin filaments (right). Bar, 200 nm. × 100,000.
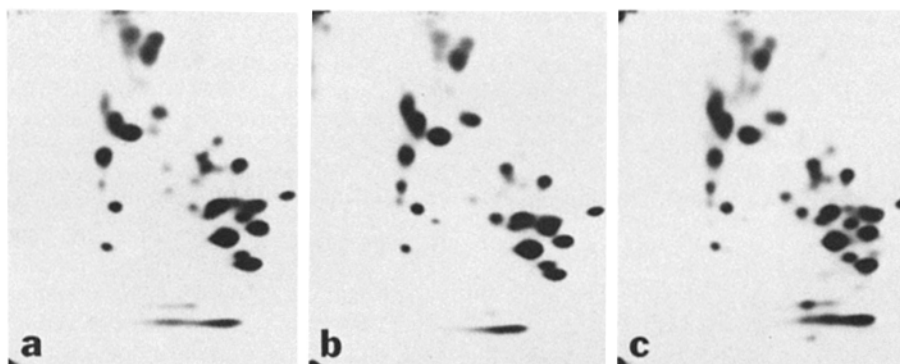

Figure 5 Two-dimensional peptide maps of ^{125}I-labeled chymotryptic peptides of human erythrocyte myosin heavy chain (a), human platelet myosin heavy chain (b), and a mixture of peptides from platelet and erythrocyte myosin heavy chains (c). Myosin from platelets and erythrocytes (50 pmol each) was denatured in 0.1% (wt/vol) SDS, radiolabeled with 1 mCi of ^{125}I by using chloramine-T as an oxidant, and electrophoresed on an SDS polyacrylamide gel. The ^{125}I-labeled heavy chains were localized by staining with Coomassie Blue, cut from the gel, and digested with 50 μg/μl alpha-chymotrypsin in 50 mM ammonium formate, 1 mM NaN$_3$ for 18 h at 37°C. The digest was lyophilized and analyzed by electrophoresis (horizontal dimension) and chromatography (vertical dimension) as described (11).

that cell shape and membrane properties were influenced by a membrane-associated actomyosin contractile apparatus. At one point, it was suggested that spectrin might be a myosin-like protein (15) and that the actin-spectrin network underlying the membrane could be the erythrocyte membrane analogue to actomyosin contractile systems in other cells (33, 34). However, the extensive work on the physical and functional properties of spectrin (18) and the recent identification of spectrin-like proteins in nonerythrocyte cells and tissues (4, 5) have made it abundantly clear that spectrin is not myosin. In this report we have established the presence in the human erythrocyte of an authentic vertebrate myosin, based on cross-reaction with affinity-purified antibodies to human platelet myosin and characterization of the structural and functional properties of the purified protein. Myosin is an endogenous component of mature human erythrocytes, based on immunofluorescence localization of myosin in all cells, and is present with respect to the actin on the erythrocyte membrane in an amount comparable to actin/myosin ratios in other nonmuscle cells (molar ratio = 80/1; see reference 29). Our work confirms and extends a preliminary report by Kirkpatrick and Sweeney (17) that erythrocyte cytosol contained myosin, and also presumably accounts for previous observations of myosin-like ATPase activities in crude preparations of spectrin from erythrocyte membranes (3, 30).

In retrospect, there are several reasons why the presence of myosin in the erythrocyte has been previously overlooked. First, the intensity of antimyosin staining of erythrocytes is considerably less intense than that of platelets or neutrophils in samples of whole blood processed for indirect immunofluorescence (V. Fowler, unpublished data). Second, the presence of interfering ATPase activities, and the similarity in molecular weight on SDS gels of the myosin heavy chain to the major cytoskeletal proteins, spectrin and ankyrin, makes it impossible to employ the usual criteria for initial identification of myosin. Finally, attention has focused on structural proteins that are tightly associated with the membrane, while the majority of the erythrocyte myosin is released into the supernatant during hemolysis and preparation of membranes under hypotonic conditions (7.5 mM sodium phosphate, pH 7.5).

In common with myosins isolated from various nonmuscle cells, the Mg^{++}-ATPase activity of erythrocyte myosin is not enhanced by the addition of rabbit skeletal muscle F-actin (19, 29). Actin activation of the Mg^{++}-ATPase activity of platelet and other cytoplasmic myosins, as well as smooth muscle myosin, occurs only after the M_r 20,000 light chain of the myosin has been phosphorylated by a calcium- and calmodulin-dependent protein kinase (1). Thus, it is possible that the M_r 19,500 light chain of erythrocyte myosin could be

TABLE I
ATPase Activity of Erythrocyte Myosin

	2 mM EDTA	10 mM CaCl₂	10 mM MgCl₂
0.5 M KCl	0.376	0.478	<0.01
0.06 M KCl	0	0.406	<0.01
0.5 M NaCl	0.118	0.204	0

ATPase activities (μmol/min per mg) were determined by using 25 μg/ml of purified erythrocyte myosin in a buffer containing 10 mM 3-(N-morpholino)propane sulfonic acid, 1 mM ATP, pH 7.0, ions as specified above, and 0.5 μCi/ml [γ-³²P]ATP. The radioactivity released as Pᵢ was determined after incubation for 15 and 30 min as described by Agre et al. (2), and was corrected for nonenzymatic Pᵢ release in the absence of myosin.

TABLE II
Quantitation of the Amount of Myosin in the Erythrocyte

	Cell equivalents (μl)*		
	1.0	1.5	2.0
Spectrin band 1 (μg)‡	0.66	0.99	1.32
Erythrocyte myosin (μg)§ heavy chain	0.030	0.048	0.066
Spectrin dimer/myosin (mol/mol)‖	33.8	31.7	30.8
Molecules myosin per cell¶	5,917	˙6,309	6,494

* Calculated from the microliters of sample electrophoresed for each determination and the volume of packed cells used to prepare the initial gel sample.
‡ Determined by the dye elution method of Fenner et al. (12) from a standard curve constructed by electrophoresing known quantities of purified spectrin in parallel with the gel samples of the erythrocytes.
§ Determined by a quantitative immunoblotting procedure as described in Materials and Methods.
‖ Calculated assuming M_r 260,000 for band 1 of spectrin and M_r 200,000 for the heavy chain of erythrocyte myosin, and that each spectrin molecule (dimer) contains one band 1 polypeptide whereas each myosin molecule contains two M_r 200,000 heavy chains.
¶ Calculated assuming 200,000 spectrin dimers per cell (5).

homologous to the M_r 20,000 regulatory light chains of other cytoplasmic myosins, and as isolated, be in the dephosphorylated state. Regulation of the level of phosphorylation of the M_r 19,500 light chain of erythrocyte myosin by a calcium- and calmodulin-dependent protein kinase, coupled with a myosin light chain phosphatase, could provide a mechanism for calcium control of erythrocyte actomyosin ATPase activity, as has been described for platelet and smooth muscle actomyosin systems (1). However, the presence in erythrocyte myosin of a light chain of apparent M_r 25,000 on SDS gels, similar to vertebrate skeletal and cardiac muscle myosins (21, 38), and unlike other previously characterized cytoplasmic or smooth muscle myosins (19, 29), suggests that other regulatory mechanisms should be considered. For example, it is possible that actin activation of the Mg^{++}-ATPase activity of erythrocyte myosin could be specific for erythrocyte membrane actin, which consists exclusively of the beta-isoelectric variant (reference 27 and V. Fowler, unpublished data), as suggested by Schrier et al. (32). Calcium regulation of erythrocyte actomyosin ATPase activity could then be provided by an actin-linked erythrocyte troponin-tropomyosin system, as discussed previously (14). Clearly, these regulatory mechanisms are not mutually exclusive, and additional myosin or actin-linked mechanisms for control of erythrocyte actomyosin ATPase activity might also exist (19, 29).

Myosin in the human erythrocyte could function, together with the membrane-associated actin protofilaments, in an actomyosin contractile apparatus responsible for ATP-dependent discocyte-echinocyte changes in cell shape and membrane properties (25, 31, 34, 37). Additionally, the passage of erythrocytes through the narrow sinusoids in the spleen may not be entirely a passive process driven by hydrostatic pressure, but may be facilitated by energy-dependent actomyosin contractions that are triggered by increases in intracellular calcium resulting from enhanced passive calcium permeability induced by physiological shear stresses (20). Such a hypothesized actomyosin contractile apparatus is probably not permanently assembled on the erythrocyte membrane, since much of the myosin is released into the supernatant after hemolysis of fresh, ATP-replete biconcave cells. The interac-

tion of myosin with the erythrocyte membrane cytoskeleton is likely to be complex and regulated at several levels: the ATP-dependent interaction of myosin heads with the actin protofilaments in the membrane skeleton (actomyosin ATPase); the self-association of molecules into bipolar filaments; and the association of filaments or individual myosin molecules via their tails to a nonactin site on the membrane. It is tempting to speculate that the increases in intracellular calcium and depletion of ATP levels that are correlated with discocyte-echinocyte shape transformations of erythrocytes (22, 24, 37) could be functionally related to assembly and activity of an actomyosin contractile apparatus on the erythrocyte membrane.

Although there is no precedent for the organization and functioning of a membrane-associated contractile apparatus, the ability of erythrocyte myosin to form bipolar filaments at the concentrations of myosin estimated to be present in the erythrocyte ($\sim$50 μg/ml)[4] raises the possibility that force production and membrane movements could result from ATP-dependent sliding of bipolar myosin filaments past antiparallel actin filaments in the membrane skeleton. The punctate character of the antimyosin staining of erythrocytes and the ratio of actin to myosin (80 monomers/myosin molecule; or 1,200 monomers/1 myosin filament of 15 monomers; reference 29) suggest that actomyosin interactions might occur at specialized sites in the membrane skeleton. Localized contractions could be transmitted through the cytoskeletal network via the multiple spectrin-band 4.1 linkages between the actin protofilaments, and tension could be exerted on the membrane via the specific association of spectrin with ankyrin. Alternatively, individual myosin molecules could be attached directly to a membrane site via their tails, leaving their heads free to interact with the actin protofilaments. The relationship of such hypothesized calcium-activated actomyosin contractions of the membrane cytoskeletal network to the previously observed inhibition of spectrin-band 4.1-actin gelation by micromolar calcium (13) is not immediately apparent. It is possible that the intracellular free calcium ion concentration could regulate spectrin-band 4.1-actin interactions concurrently with actomyosin contractions in a membrane-associated counterpart of the solation-contraction coupling mechanism that has been proposed to explain amoeboid movements (8, 9, 16). Clearly, evaluation of these ideas will require extensive biochemical and ultrastructural investigation of the interaction of myosin with the membrane and with the actin filaments in the membrane skeleton. In particular, the relationship of actomyosin interactions to the spectrin-band 4.1-actin linkages in the membrane cytoskeleton, as well as the locations and associations of potential regulatory proteins such as tropomyosin (14) or troponins, will need to be defined.

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[4] Calculated from the number of copies of myosin per cell (6,000, see Results) and a cell volume of 85 μm^3.

Note added in Proof: In a recent experiment by Albert J. Wong, Daniel P. Kiehart, and Thomas D. Pollard (*J. Biol. Chem.*, in press), phosphorylation of the M_r 19,500 light chain was observed to be correlated with the actin-activated ATPase activity of erythrocyte myosin.

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