Myosin in Adult and Neonatal Human Erythrocyte Membranes

By Lisa M. Matovcik, Ute Gröschel-Stewart, and Stanley L. Schrier

The heavy chain of myosin can be detected in human red cell membranes by immunoblot analysis with antiplatelet myosin antibodies. Neonatal red cell membranes have more immunoreactive myosin than adult membranes. Membranes from young adult red cells contain more immunoreactive myosin than membranes from old adult red cells. In contrast, young and old neonatal red cells have equivalent amounts of myosin. Erythrocyte myosin is present in a membrane fraction enriched in integral membrane proteins but is not found in cytoskeletal preparations.

Materials and Methods

Materials. All materials purchased were of reagent grade or better. Stratchan was prepared as previously described. Equipment for SDS-PAGE and Western blotting, nitrocellulose paper, and the chemicals acrylamide, bis-acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), and Temed were purchased from Biorad Laboratories (Richmond, Calif). Horseradish peroxidase conjugated swine antirabbit immunoglobulins and rabbit peroxidase-antiperoxidase were purchased from Dako Corporation (Santa Barbara, Calif). Swine serum was purchased from Gibco Laboratories (Grand Island, NY). The following reagents were purchased from Sigma Chemical Co (St Louis, Mo): alpha-cellulose, cellulose (Sigmacl-50), Tris-adenosine 5'-triphosphate, calcium ionophore A23187, Nonidet P-40 (NP40), 4-chloro, 1-napthol, and Triton X-100.

Methods. Protein purification and antibody production. Spectrin and actin were purified from outdated human erythrocytes. Protein 2.1 was purified from freshly drawn adult and neonatal blood. Platelet myosin was purified as described by Peleg et al. Proteins were judged to be pure by SDS-PAGE electrophoresis. Protein concentrations were determined either by the method of Lowry or Bradford.

Antibodies to platelet myosin were raised in rabbits using two platelet myosin preparations, platelet membrane myosin, and platelet cytoplasmic myosin. These antibodies have been previously characterized.

Erythrocyte membrane preparation and fractionation. Blood was drawn from normal adult volunteers and from the placental vessels of premature and term newborns at the time of delivery according to established protocols of the Stanford Committee for the Protection of Human Subjects in Research. Platelets were removed by defibrination, or removed along with the white cells by passage over a column of 50% cellulose, 50% alpha-cellulose. This removed the white cells and platelets to below the threshold of detectability of the Coulter Model S Plus 4 or S Plus 2. Red cells were separated on discontinuous Stracan density gradients as previously described. Red cell membranes were prepared by the method of Dodge. Inside-out vesicles were prepared by the method of Steck and Kant, and both isotonic and hypertonite Triton cytoskeletons by the method of Sheetz.

Ca2+ loading and metabolic depletion. Calcium was introduced into adult and neonatal red cells with the calcium ionophore A23187. Red cells were incubated for 30 minutes at 37 °C at a hemocrit of approximately 0.15 in 0.154 mol/L NaCl with either 100 μmol/L CaCl2 or 1.0 μmol/L A23187 or both. In the ionophore controls, 1.0 mmol/L EDTA was added to ensure that small amounts of contaminating Ca2+ in the water would not enter RBC and confuse the results. Adult and neonatal red cells were depleted of ATP by gently

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rocking the whole blood in the sterile collection tubes in a horizontal position for 24 hours. After the incubations, the red cell ATP content was measured in an aliquot of cells, an aliquot was fixed in glutaraldehyde and later examined by phase microscopy, and white ghosts were prepared from the rest of the red cells.

**Electrophoresis and Western blotting.** Gel electrophoresis was performed by the method of Laemmli on convex gradient gels of 4% to 14% acrylamide with a 15 mL mixing volume. For antibody staining the gels were transferred at 50 volts for approximately 14 hours to nitrocellulose paper, then incubated at 37 °C for 1 hour in 3% BSA, 1% swine serum (blocking solution). The primary antibody was diluted in blocking solution to a concentration of 50 to 100 μg protein/mL and incubated with the papers at 4 °C for 18 to 24 hours, washed extensively in PBS pH 7.4, then PBS with 0.05% NP40, again with PBS, then incubated with horseradish peroxidase labeled swine antirabbit immunoglobulin diluted 1:100 in blocking solution at room temperature for 90 minutes and the wash procedure repeated. The papers were stained with the chromagen 4-chloro-1-napthol, 3 mg/mL in methanol (1 part), 154 mmol/L NaCl, buffered with 25 mmol/L Tris, pH 7.4 (5 parts), 3% H2O2 (0.2 parts). Color development was complete in 2 to 10 minutes.

**ATPase assays.** Mg²⁺-ATPase and actin-activated ATPase assays were performed as previously described. Ca²⁺. Mg²⁺-ATPase was assayed under conditions identical to those used for Mg²⁺-ATPase with 120 μmol/L calcium chloride present. [Gamma-32P]-ATP cleavage in all three assays was performed as previously described. Ca²⁺. Mg²⁺-ATPase and actin-activated ATPase assays were performed as previously described. The nonimmune immunoglobulin does not react with any of the proteins under study.

Two of the major proteins of the erythrocyte membrane, protein 2 (spectrin beta chain), and protein 2.1 (ankyrin), have a molecular weight similar to the myosin heavy chain and could obscure the myosin band on gels stained to visualise protein. The myosin heavy chain can be shown to be a protein separate from both proteins 2 and 2.1. On 4% to 14% gradient gels transferred to nitrocellulose and stained with Amido Black 2, protein 2 is distinctly above the immunoreactive band on an identical paper that is stained with antiplatelet myosin. Likewise, overexposure of the antibody stained papers to the chromagen results in a faint background staining of all the proteins on the gel, and protein 2 can easily be distinguished from the darkly stained myosin. In order to determine if the antibody-stained protein is protein 2.1 we tested protein 2.1 purified from both adult and neonatal red cell membranes in the Western blot system and found that the purified protein 2.1 did not react with the antiplatelet myosin (data not shown).

Care was taken in the preparation of the red cells from which the ghosts were made to remove white cells and platelets, both of which contain myosin. The number of white cells and platelets remaining in the red cell preparations after filtration on the cellulose: alpha-cellulose column was always below the threshold of the electronic cell counter used to detect them. No white cells or platelets were visible upon microscopic examination of stained smears. We tested samples containing increasing, known numbers of platelets and white cells to determine the sensitivity of the antimyosin to contaminating cells under the Western blot conditions used in this study. The antiplatelet myosin can detect platelet myosin from a minimum of 2 x 10⁴ platelets per gel lane. This is three orders of magnitude more platelets than could have contaminated the 40 μg of ghost protein routinely applied to the gels given the manufacturer's stated detection threshold of the Coulter counter. Likewise, the number of white cells that contain the threshold of myosin the antibody is able to detect lies between 10 and 100 times the theoretical maximum number of white cells in the ghost sample loaded on the gel.

**Neonatal red cell membranes have greater reactivity to platelet antomyosin than adult red cell membranes.** Figure 2 compares the antiplatelet myosin binding of white ghosts prepared from adult and neonatal red cells. Although there are no differences in the protein content of adult and neonatal red cells detectable by visual examination of Coomassie blue stained gels (panel A), the neonatal membrane contains more immunoreactive myosin than the adult membrane (panel B). Incubating adult and neonatal ghosts together at room temperature does not inhibit the antimiysin cross reactivity in the neonatal ghosts. The combination of adult and neonatal ghosts reacts with platelet antomyosin on a Western blot to approximately the same degree as the sum of the adult and neonatal membrane tested separately (data not shown).
To determine whether enhanced antiplatelet myosin immunoreactivity is a feature of younger cells in general or only neonatal cells we separated both adult and neonatal red cells according to their density on discontinuous Stractan gradients. In general, light cells are young and dense cells are old. Figure 3 shows the Coomassie blue stain, panel A, and the corresponding antiplatelet myosin Western blot, panel B, of young, middle-aged, and old red ghosts prepared from red cells isolated from adults (lanes 1–3), and newborns (lanes 4–6). In lane 1 (young), 2 (middle-aged), and 3 (old) adult red cells, the intensity of the antimyosin staining decreases with cell age. In lane 4 (young), 5 (middle-aged), and 6 (old) neonatal red cells, the intensity of antimyosin staining is greater in all three fractions than in the corresponding adult fraction. No decrease with age can be observed in neonatal red cells.

ATP depletion and increase in the intracellular Ca\textsuperscript{2+} level have no effect on the amount of myosin in the membrane. In order to determine conditions that might alter the amount of myosin that is bound to the membrane, we allowed Ca\textsuperscript{2+} to enter red cells with the ionophore A23187. In the presence of 100 \( \mu \)mol/L CaCl\textsubscript{2} and 1.0 \( \mu \)mol/L A23187, both the adult and neonatal red cells had a spherocinhydrocytic morphology. This treatment reduced the ATP levels in these red cells to one half or less of their normal values (Table 1). Incubation of the red cells with 100 \( \mu \)mol/L-Ca\textsuperscript{2+} without ionophore did not result in an echinocytic shape change or lowered ATP levels.

Sterile incubation of whole blood at 37°C for 24 hours depleted the red cell ATP content to barely detectable levels (Table 1). This incubation also resulted in extensive formation of echinocytes in both adult and neonatal red cells after depletion, with a few discocytes left. White ghosts were prepared from both Ca\textsuperscript{2+}-loaded and ATP-depleted red cells. The Western blot of the Ca\textsuperscript{2+}-loaded and ATP-depleted red cell membranes is shown in Fig 4. Neither treatment altered the amount of immunoreactive myosin present in either the adult or neonatal ghost preparation.

Effect of Mg\textsuperscript{2+} and Mg\textsuperscript{2+}-ATP on the membrane-associated myosin. The addition of 1.0 mmol/L MgCl\textsubscript{2} to the white ghost lysis buffer results in more tropomyosin associating with the membrane preparation. To determine whether Mg\textsuperscript{2+} resulted in more myosin association as well, white ghosts were prepared with 1.0 mmol/L MgCl\textsubscript{2} added to the lysis buffer (5 mmol/L PO\textsubscript{4}, pH 8.0). No difference in the intensity of antimyosin staining was detected as a result (data not shown).

Since ATP affects the association of actin with myosin, we studied the effect of adding Mg\textsuperscript{2+}-ATP to the lysis buffer. White ghosts were prepared with 1 mmol/L MgCl\textsubscript{2} and 1 mmol/L Tris-ATP in the lysis buffer, and each of the three washes. Western blot analysis with anti-platelet myosin did not detect any differences between ghosts prepared with or without Mg\textsuperscript{2+}-ATP (Fig 4, Panels A & B, lanes 2).

Myosin is present in inside-out vesicles, but not in Triton cytoskeletons. In order to localize the membrane domain that contains myosin we fractionated the red cell membrane into inside-out vesicles and Triton cytoskeletons (see Methods). Inside-out vesicles (IOVs) are depleted in cytoskeletal proteins and enriched in integral membrane proteins such as protein 3 and the glycoporphins (Fig 5A, lanes 2 and...
Table 1. ATP Levels in Ca²⁺-Loaded and ATP-Depleted RBC

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adult (µmol/mL RBC)</th>
<th>Neonate (µmol/mL RBC)</th>
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<tbody>
<tr>
<td>Untreated RBC</td>
<td>1.80</td>
<td>1.64</td>
</tr>
<tr>
<td>CaCl₂ + A23187</td>
<td>0.61</td>
<td>0.80</td>
</tr>
<tr>
<td>Sterile depletion</td>
<td>0.01</td>
<td>0.06</td>
</tr>
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6). Triton cytoskeletons are enriched in the cytoskeletal proteins spectrin, actin, and protein 4.1. When isolated under isotonic conditions, Triton cytoskeletons also contain small amounts of various other membrane proteins (Fig 5A, lanes 3 and 7), but Triton cytoskeletons isolated under hypertonic conditions (0.6 M KCl) retain very little of proteins 3, 2, 1, or 4.2, glycoporphins, or other minor membrane components and are composed almost solely of spectrin, actin, and protein 4.1 (Fig 5A, lanes 4 and 8). The presence of myosin was monitored by Western blot analysis and an assay for actin-activated ATPase activity was performed on each fraction.

The Western blot analysis with antiplatelet myosin is shown in Fig 5B. The red cell membrane myosin remains with the inside-out vesicles and is not present in Triton cytoskeletons prepared either isotonically or hypertonically.

ATPase activities of adult and neonatal red cell membranes. Red cell ghosts contain several well-characterized ATPase activities, among them an actin-activated Mg²⁺-ATPase which is an identifying characteristic of myosin. We compared the activity of Mg²⁺-ATPase, actin-activated Mg²⁺-ATPase, and Ca²⁺, Mg²⁺-ATPase in adult and neonatal red cells (Table 2) to see if the enzyme activities correspond to the degree of antymosin reactivity. If myosin is present in the isolated membrane preparation along with the regulatory factors necessary for activity, then the neonatal red cell membrane would be expected to have more actin-activated Mg²⁺-ATPase activity than adult membranes.

Table 2 compares the Mg²⁺, actin-activated, and Ca²⁺, Mg²⁺-ATPase activities of adult and neonatal cell membranes. There is no significant difference between the baseline level of total Mg²⁺-ATPase in freshly prepared neonatal and adult white ghosts. This Mg²⁺-ATPase activity is age-
dependent; ghosts from young red cells have more activity than ghosts from old cells. The mean population of neonatal red cells has the same amount of Mg\textsuperscript{2+}-ATPase activity as adult red cells but the age dependence is more pronounced between the youngest and oldest adult cells (data not shown). Addition of erythrocyte F-actin (but not rabbit muscle F-actin) results in an increase in a component of the Mg\textsuperscript{2+}-ATPase activity to approximately the same extent in neonatal and adult red cells. Actin-activated Mg\textsuperscript{2+}-ATPase shows no age dependence in adult or neonatal red cells. The Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-ATPase activity does not differ significantly in adult and neonatal red cell membranes.

Actin-activated Mg\textsuperscript{2+}-ATPase activity was measured in ghosts, IOVs, and Triton cytoskeletons and compared to the amount of immunoreactive myosin. Although IOVs retain 56% and Triton cytoskeletons 12% of the total Mg\textsuperscript{2+}-ATPase activity of ghosts per weight membrane protein, neither membrane fraction has any measurable actin-activated Mg\textsuperscript{2+}-ATPase activity.

The myosin immunoreactivity and actin-activated ATPase activity do cosediment when ghosts are extracted with 0.6 mol/L KCl, a method used to extract myosin from smooth muscle and nonmuscle cells. Red cell myosin is not soluble in 0.6 mol/L KCl. The supernatant neither contains actin-activated ATPase activity nor reacts with antiplatelet myosin in the Western blot system. The pellet does contain immunoreactive myosin, and the actin-activated ATPase activity can be recovered quantitatively in this fraction (data not shown).

An experiment testing whether antiplatelet myosin could inhibit the actin-activated ATPase activity of ghosts was not conclusive because, although equimolar amount of immunoglobulin to membrane protein did effectively eliminate ATPase activity in leaky ghosts, the control antibody to a cytosolic-facing membrane protein, spectrin, had the same effect.

**DISCUSSION**

Myosin has only recently been identified in the human red cell,\textsuperscript{1-37} probably because it is present in very small amounts, and because human red cell membrane myosin requires an extraction procedure that differs from methods routinely used to purify uterine and platelet myosin.\textsuperscript{30-38} Tryptic peptide analysis shows that erythrocyte myosin is very similar to platelet myosin.\textsuperscript{3}

In this report we relied on immunoblot analysis to establish the presence or absence of red cell membrane myosin and to indicate the relative amount present. While it would have been preferable to do a chemical extraction and purification, such experiments are not technically possible because of the small amount of myosin present in the red cell: about 6,000 copies per cell.\textsuperscript{3,37} The amounts of blood required (1 to 2 units)\textsuperscript{30} cannot be obtained at all from neonates, and would be difficult to obtain from density-separated populations of red cells and from fractions of integral membrane proteins and cytoskeletal preparations.

Our results (Figs 2, 3, 4, and 5) show that more immunoreactive myosin is present in the membranes of neonatal than in adult red cells. The antiplatelet membrane myosin gave the same results as anticytoplasmic myosin although the two immunogens are distinct.\textsuperscript{35} Neonatal red cells may contain more myosin or more of the total red cell myosin may be associated with the membrane. Erythrocyte myosin may behave similarly to platelet\textsuperscript{39} or brush border epithelial cell\textsuperscript{40} myosin and be present in an equilibrium between the cytoplasm and the membrane which depends on its state of phosphorylation. A third possibility is that there is a fetal myosin isozyme that is more immunoreactive with these particular antibodies.

Since neonatal erythrocytes are in general a younger cell population than adult erythrocytes, we determined the contribution of cell age by separating adult and neonatal red cells into density, and thus probably age-dependent, populations and compared the amount of antiplatelet myosin reactivity in each. The amount of immunoreactive membrane myosin decreases as the adult erythrocyte ages. Immature red cells remodel their membranes to a greater degree than mature red cells; myosin may be involved in this remodeling. No decrease in immunoreactivity with age was observed in neonatal red cell membranes. The interpretation of this result is complicated by the fact that the older neonatal red cells were produced by a less developmentally mature fetus than the younger neonatal red cells.

The amount of red cell membrane-associated myosin may be controlled by physiologic events as it is in other tissues. Differences in these control mechanisms could account for the differences in immunoreactive myosin seen in adult and neonatal red cell membranes. In other tissues the amount of Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, or ATP can influence the extent of membrane association by influencing the state of myosin phosphorylation. However, neither ATP depletion nor Ca\textsuperscript{2+} accumulation nor Mg\textsuperscript{2+}-ATP addition to lysis buffer altered membrane myosin retention in adult or neonatal RBC membranes (Fig 4).

Since actin is an important component of the red cell membrane cytoskeleton, we assumed that cytoskeletal preparations would be enriched in immunoreactive myosin as well, whereas the inside-out vesicles, stripped of their skeletal proteins and containing the integral transmembrane proteins, would have little myosin. In fact, the opposite was the case, and equally so in neonatal and adult membrane preparations (Fig 5).

Actin-activated ATPase activity, which serves to identify myosin, is present in red cell ghosts. Estimates of the number of molecules of myosin per red cell, and the percent of myosin that is membrane-associated\textsuperscript{37} suggest that about 1/1000 of the adult red cell membrane protein weight is myosin. The Mg\textsuperscript{2+}-ATPase activity simulated by human erythrocyte actin can then be calculated to be roughly 8 μmol P:\textsubscript{i}/mg myosin/min, a value considerably higher than results obtained using rabbit muscle actin.\textsuperscript{37} However in our prior study we found that rabbit muscle actin induced very little actin-activated Mg\textsuperscript{2+}. ATPase activity compared to human erythrocyte actin. We then sought to determine if this enzyme activity parallels the immunoblot data. Both anti-myosin reactivity and actin-activated Mg\textsuperscript{2+}-ATPase were present in the residual pellet of a 0.6 mol/L KCl extraction, but the enzyme activity was lost in the IOV and the Triton
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cytoskeleton preparations. If antiplatelet myosin reactivity
and actin-activated Mg\(^{2+}\)-ATPase activity are detecting the
same protein, then modulators of the enzyme activity could
be lost when the membrane is disrupted. The ATPase of
purified nonmuscle myosins is not activated by actin unless
necessary cofactors such as myosin light chain kinase are
present.\(^{41}\) For example, platelets\(^{16}\) and macrophages\(^{42}\) myosin
are both regulated by phosphorylation of the 20 kDa light
chain by a myosin light chain kinase. Neonatal ghosts and
IOVs contain more immunoreactive myosin than adult
ghosts and IOVs but do not have substantially more actin-
activated Mg\(^{2+}\)-ATPase activity. The reasons for this dis-
crepancy may become clear when the regulation of enzymic
activity of erythrocyte myosin is understood.

The discovery of myosin in red cells\(^{27}\) establishes the fact
that a potentially complete contractile system, including
myosin, actin, and tropomyosin, is present at the membrane.
The role of the contractile apparatus is currently unknown.
Very likely it is functional in motile red cell progenitors\(^{1}\)
where it may be involved actively in nuclear extrusion or
receptor-mediated endocytosis. Reticulocytes are formed
when the orthochromic normoblast ejects the nucleus with
dynamic contractions.\(^{43}\) Erythroblasts undergo receptor-
mediated endocytosis to internalize ligands necessary for cell
growth. Contraction of the actomyosin complex has recently
been implicated in insulin receptor endocytosis in human
lymphoblastoid cells.\(^{5}\) The fact that myosin is found asso-
ciated with integral red cell membrane protein in IOVs and
not with actin in the cytoskeleton also raises the hypothesis
that under certain unknown circumstances the interaction
between myosin and actin could serve to link cytoskeletal
components with integral proteins in a manner that supple-
ments the spectrin-ankyrin-protein 3 interaction.

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