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hsp 70-Like Protein in Rhesus Erythrocyte Cytosol and Its Interactions with Membrane Skeleton under Heat and Pathologic Stress*

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Tanima Gudi‡ and Chhitar M. Gupta§

From the Institute of Microbial Technology, Chandigarh-160014, India

The rhesus erythrocytes were examined for the presence of protein(s) similar to the 70-kDa class of heat shock proteins (hsp 70). Also, interactions of these proteins with the erythrocyte membrane were studied under heat stress. These cells in their cytosol contained at least two proteins of about 70 kDa molecular mass; one of which closely resembled the hsp 70 family of proteins. This protein under normal conditions localized mainly in the cytosol, but it had a strong tendency to bind the membrane under heat stress. The binding was almost exclusively restricted to the membrane skeleton and seemed to involve primarily the hydrophobic interactions. A 70-kDa protein immunologically similar to the above protein(s) was detected also in the membranes of rhesus erythrocytes harboring the schizont stage of the simian malarial parasite *Plasmodium knowlesi*. From these results, we conclude that hsp 70-like proteins in the mature mammalian erythrocytes could perhaps play an important role in protecting the cells under stress by stabilizing the membrane skeleton through their interactions with skeletal proteins.

Virtually all types of cells respond to unfavorable conditions, such as heat shock, metabolic starvation, infection, etc., by synthesizing in large quantities a set of proteins, called heat shock proteins (hsp).[1] Also, proteins similar to hsp exist in the normal cells. Both the stress-induced and constitutively expressed proteins protect the cells from the deleterious effects of heat and other stresses by catalyzing the repair of the partially damaged protein structures (reviewed in Refs. 1–3). Of these proteins, the hsp 70 family of proteins constitutes the major group, accounting for about 1% of the total cellular protein even under normal growth conditions (1–6). All known members of this family are ATP-binding acidic proteins which invariably bind the partially unfolded protein structures, thus preventing further unfolding and consequently the aggregation of cellular proteins at high temperatures (1–7). These proteins have highly conserved structures and are present in a variety of cells (1–6), including erythrocytes (8).

Mature mammalian erythrocytes contain cytoskeleton (called "membrane skeleton") which is totally associated with the inner surface of the membrane bilayer. The membrane skeleton is primarily composed of three major, *viz.* spectrin, actin and polypeptide 4.1, and several minor proteins (9). Among these, spectrin is the major protein which alone accounts for about 75% of the total skeletal protein mass (10). This protein is comprised of two subunits, *viz.* α (260 kDa) and β (225 kDa), and is known to undergo unfolding at higher temperatures (11, 12). It has been shown that irreversible structural changes occur in spectrin by subjecting the intact human erythrocytes (13), erythrocyte ghosts (11, 12), or pure protein (14) to heating at about 50 °C.

Earlier studies have suggested that the rhesus erythrocyte spectrin is more susceptible than the human erythrocyte spectrin to the heat-induced structural changes, under identical conditions (15). Since the normal body temperature of rhesus monkeys (*Macaca mulatta*) is about 2–3 °C higher than the normal human body temperature (16), further rise in the body temperature during fever or other pathologic conditions could affect the spectrin structure in the rhesus erythrocytes. To investigate this problem, we considered it of interest to look for the presence of hsp-like protein(s) in the rhesus erythrocytes and also to analyze the interactions, if any, of such protein(s) with the membrane skeleton under heat stress. Here, we report that these erythrocytes do contain an hsp 70-like protein which specifically binds the membrane skeleton at higher temperatures (44 °C) and also during infection of these cells by the malarial parasite *Plasmodium knowlesi*.

EXPERIMENTAL PROCEDURES

Materials—PMSF, DTT, EGTA, EDTA, HEPES, Mg^{2+}-ATP, bovine serum albumin, adenosine, acrylamide, bisacrylamide, ammonium persulfate, Nonidet P-40, β-mercaptoethanol, molecular weight markers, Triton X-100, anti-rabbit IgG-peroxidase conjugate, anti-mouse IgG-peroxidase conjugate, 4-chloro-1-naphthol, glycylglycine, DE52, hydroxyapatite, ATP-agarose (C_8-linked), and ampholines pH 3–10 and pH 4–6.5 were obtained from Sigma. Anti-hsp 72/73 monoclonal antibody (hsp 70 mAb) was procured from Stress-Gen, Canada. Nitrocellulose membranes were from Bio-Rad.

Erythrocytes—Blood was drawn from healthy human subjects and normal rhesus monkeys in glass tubes containing acetate/citrate/dextrose. Plasma and buffy coat were removed by washing the cells with phosphate-buffered saline (7.5 mM phosphate containing 150 mM NaCl, pH 7.4; PBS).

Heat Treatment—Rhesus/human erythrocytes were suspended to 10% hematocrit in 10 mM glycylglycine containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, and 1 mM adenosine, pH 7.4, and subjected to heating at 44 °C or as stated otherwise under an atmosphere of humidified N_2. The cells were harvested by centrifugation.

Erythrocyte Membranes—Erythrocyte membranes were prepared according to Fairbanks *et al.* (17). All operations were carried out at 0–4 °C, unless otherwise stated. Washed erythrocytes were lysed with

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§ To whom correspondence and reprint requests should be addressed.

[1] The abbreviations used are: hsp, heat shock protein(s); hsc, heat shock cognates; hsp 70 mAb, monoclonal antibody specific to hsp 72/73; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; RBC, red blood cells.

40 volumes of ice-cold 7.5 mM sodium phosphate, pH 7.5, containing PMSF (1 mM), pepstatin A (2 μg/ml), and leupeptin (2 μg/ml) as protease inhibitors. The membranes were collected by centrifugation at 30,000 × *g* for 20 min and washed with the same buffer 3–4 times.

Erythrocyte Cytosol—Erythrocytes were lysed hypotonically with 10 volumes of 7.5 mM sodium phosphate, pH 7.4, containing 20 μg/ml PMSF. The membranes were separated by centrifugation at 30,000 × *g* (20 min). The supernatant (cytosol) was collected carefully for isolation and purification of 70-kDa proteins.

Triton Shells—Triton-insoluble membrane skeletons (Triton shells) were prepared from the erythrocyte membranes as described earlier (18). The membranes were treated with an equal volume of 24 mM HEPES buffer, pH 7.0, containing 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.5 mM DTT, and 15% Triton X-100 at ice-cold temperature. Portions of this lysate were layered onto 30% sucrose columns in centrifuge tubes containing 24 mM HEPES, 600 mM KCl, 0.5 mM DTT, and 0.5 mM EDTA, pH 7.0. The tubes were centrifuged at 20,000 × *g* for 35 min. The pellets were washed with ice-cold isotonic saline.

Anti-rhesus Erythrocyte 70-kDa Cytosolic Protein Antibodies—Antibodies to monkey erythrocyte 70-kDa cytosolic protein were raised in rabbits after elution of the protein from preparative SDS-polyacrylamide gels.

Membranes of Rhesus Erythrocytes Infected with Schizont Stage of Plasmodium knowlesi—Synchronous infections of *P. knowlesi* were maintained in healthy rhesus monkeys (19). The monkeys were bled at ~30% parasitemia, the schizont-infected cells were separated, and the parasite-free erythrocyte membranes were isolated by differential centrifugation essentially according to Joshi *et al.* (19).

Cytosol Interaction with Membrane Skeletons—The cytosol obtained as above was made isotonic by adding to it solid NaCl. PMSF and DTT were also added to obtain 1 mM and 0.5 mM final concentrations, respectively. The cytosol was divided into four equal parts, out of which two parts were preheated at 44 °C for 1 h. Triton shells prepared from the normal erythrocyte membranes were divided into five equal parts. The first and second parts were mixed with the normal unheated cytosol; one was incubated at 0 °C and other at 44 °C for 1 h. The third and fourth parts were mixed with the preheated cytosol; one was incubated at 0 °C and other at 44 °C for 1 h. The fifth part was mixed with PBS and heated at 44 °C (1 h). All the mixtures prior to incubation were flushed with N_2. After incubation, the mixtures were centrifuged at 20,000 × *g* for 30 min. The pellets and supernatants were analyzed by SDS-PAGE. Equal amounts of protein were loaded in cases of pellets, and equal volumes of samples were analyzed in cases of the supernatants. The 70-kDa protein-to-spectrin ratio was calculated from the densitometric scans of Coomassie Blue-stained SDS-polyacrylamide gels. Release of spectrin into the medium during heating of the Triton shells with cytosol or buffer was calculated by scanning the spectrin monomer bands released in the supernatant.

Protein Purification—Rhesus erythrocyte 70-kDa cytosolic proteins were purified by adopting the procedure of Davis and Bennett (8) described for purification of clathrin uncoating ATPase from the human erythrocytes. The first step in the purification scheme involved an anion exchange chromatography of the cytosol over DEAE-cellulose (DE52), while the second step involved adsorption chromatography on a hydroxyapatite column. The 70-kDa protein thus obtained was over 80% pure, which was further purified either by high pressure liquid chromatography (Pharmacia LKB Biotechnology Inc. HPLC System) using a TSK-SWG-300 gel filtration column or by affinity chromatography on ATP-agarose.

Electrophoresis—Protein compositions in erythrocytes, erythrocyte membranes, cytosol, spectrin-actin extracts, and Triton shells were determined by SDS-PAGE according to Hubbard and Lazarides (20) using 5% acrylamide as stacking and 10% acrylamide as running gels. The gels were stained with Coomassie Blue and scanned on a Shimadzu dual wavelength scanner CS-910 at 560 nm. In some cases, the protein bands were visualized also by the silver staining (21).

Immunoblotting—The protein samples were first subjected to SDS-PAGE and then transblotted on the nitrocellulose membranes essentially according to Towbein *et al.* (22). The transfer was carried out at 50 V for 3 h using a Bio-Rad Transblot cell. One strip of the nitrocellulose membrane was stained with Amido black to check the transfer, and the other was used for immunostaining. Free sites on the nitrocellulose membrane were blocked with 5% bovine serum albumin. It was incubated with hsp 70 mAb or anti-rhesus erythrocyte 70-kDa protein antisera (1:1000 dilution) and, after washing with 20 mM Tris buffer containing 500 mM NaCl and 0.2% Tween 20 (pH 8.0), further incubation was done with anti-mouse IgG-peroxidase conjugate (in the case of monoclonal antibody) or anti-rabbit IgG-peroxidase conjugate (in the case of polyclonal antibody). The strips were washed extensively and then developed with 4-chloro-1-naphthol (23).

Isoelectric Point Determination—The isoelectric point of the 70-kDa protein was determined essentially by following the procedure of O'Far-

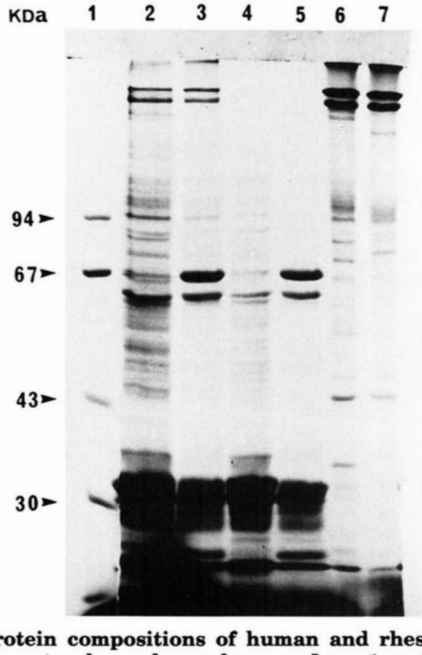

FIG. 1. **Protein compositions of human and rhesus erythrocytes, their cytosols, and membranes.** *Lane 1*, molecular mass markers; *lane 2*, human RBC; *lane 3*, rhesus RBC; *lane 4*, human RBC cytosol; *lane 5*, rhesus RBC cytosol; *lane 6*, human RBC membrane; *lane 7*, rhesus RBC membrane.

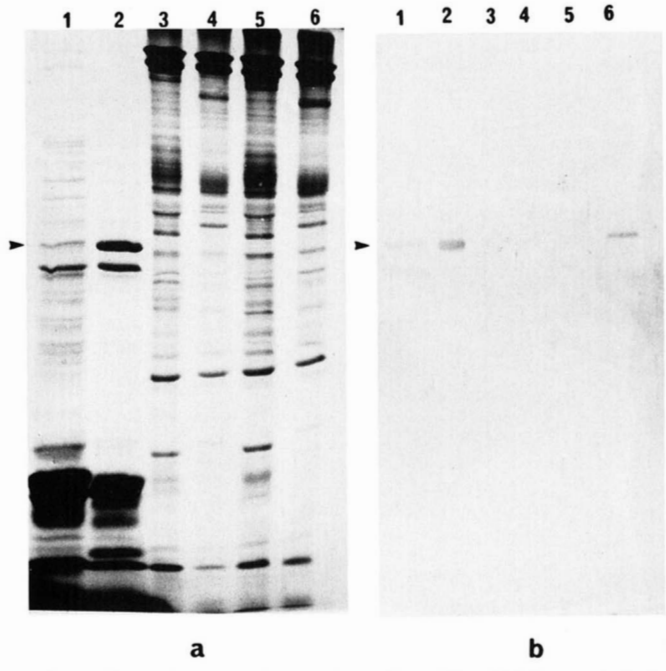

FIG. 2. **Cytosol-to-membrane transfer of the 70-kDa protein in erythrocytes under heat stress.** Both the human and rhesus erythrocytes were subjected to heat stress by incubation at 44 °C for 1 h. The cells were lysed, and the cytosolic and membrane fractions were prepared. These were subjected to SDS-PAGE (*panel a*), transferred to nitrocellulose paper, and immunoblotted with rabbit anti-rhesus RBC 70-kDa cytosolic protein primary antibodies and a peroxidase-conjugated secondary antibody (*panel b*). *Lane 1*, normal human RBC cytosol; *lane 2*, normal rhesus RBC cytosol; *lane 3*, normal human RBC membrane; *lane 4*, normal rhesus RBC membrane; *lane 5*, membranes of heat-stressed human RBC; *lane 6*, membranes of heat-stressed rhesus RBC. *Arrow* marks the position of the 70-kDa cytosolic protein that tends to associate with the membrane under heat stress.

rell (24), using a mixture of pH 4–6.5 ampholine (4 parts) and pH 3–10 ampholine (1 part).

RESULTS

The rhesus erythrocyte is different from the human erythrocyte in that it contains large quantities of a protein of about 70 kDa molecular mass, which appears to be only a minor component of the human cells (Fig. 1). This protein is localized mainly in the cytosol, but a very minor band corresponding to this protein could also be detected in the erythrocyte membrane. That the 70-kDa protein seen in the normal rhesus erythrocyte membranes is similar to that present in the cytosol was ascertained by examining the cross-reactivity of the membrane 70-kDa protein to the polyclonal antibodies raised against the rhesus erythrocyte 70-kDa cytosolic protein in rabbits. These antibodies, besides recognizing the rhesus erythrocyte membrane 70-kDa protein, also reacted with two human erythrocyte cytosolic proteins of about 60 kDa and 70 kDa molecular mass (Fig. 2). The quantities of the 70-kDa protein in the rhesus erythrocyte membrane were significantly increased when the cells were subjected to heat shock at 44 °C for 1 h. However, no such cytosol-to-membrane transfer could be detected, after probing with the polyclonal antibody, in the human red cells under identical conditions (Fig. 2). This apparent lack of the 70-kDa protein transfer may be attributed to much smaller quantities of this protein present in the human erythrocytes, in addition to the poor cross-reactivity of the human 70-kDa protein to the polyclonal antibody.

The cytosol-to-membrane 70-kDa protein transfer in the monkey erythrocytes depended on the temperature at which the cells were subjected to the heat stress (Fig. 3). In this case, the transfer was probed by both the anti-rhesus erythrocyte 70-kDa polyclonal antibody and hsp 70 mAb. The extent of this transfer appeared to increase with the temperature when probed by the polyclonal antibody (data not shown), but it seemed to remain unaltered after 46 °C, when probed with hsp 70 mAb (Fig. 3*b*). As may be seen in Fig. 3*a*, along with the 70-kDa protein, several other cytosolic proteins were also transferred to the membrane at higher temperatures (46 °C). Unlike this finding, nearly selective transfer of the 70-kDa protein was observed when the cells were subjected to thermal stress at 44 °C at least up to 4 h (Fig. 4). In this case, the extent of transfer increased with the duration of the heat treatment.

To localize the 70-kDa protein in the membranes of the heat-treated cells, we prepared the membrane skeletons (Triton shells) from both the normal and heated erythrocytes and then examined their protein compositions by SDS-PAGE. Fig. 4 (*lanes 6–9*) shows that unlike the normal erythrocyte membrane skeleton, the membrane skeletons of the heated erythrocytes, besides containing spectrin, actin and polypeptide 4.1, also contained significant quantities of the 70-kDa protein, which increased by increasing the duration of the heat treatment. Most of the 70-kDa protein transferred from the cytosol to the membrane during heating was localized in the membrane skeleton, as we observed similar 70-kDa protein-to-spectrin ratios in the membranes and Triton shells prepared from the heated erythrocytes (Table I).

To further confirm whether the rhesus erythrocyte 70-kDa protein was selectively transferred from the cytosol to the membrane skeleton under heat stress, we have studied the interaction of cytosol with the Triton shells prepared from the normal erythrocyte membrane. The shells were incubated with the cytosol or PBS at 44 °C for 1 h as described under "Experimental Procedures," and the protein transfer was ascertained by measuring the 70-kDa protein-to-spectrin ratio in the Triton shell pellets obtained after removing the cytosol by centrifugation. Fig. 5 shows that appreciable amounts of the 70-kDa protein were transferred to the Triton shells during the heat treatment. These amounts further increased by incubating the Triton shells with preheated (44 °C, 1 h) cytosol under identical conditions (Table II). The effect of the heat stress on the stability of the Triton shells was evaluated by measuring the amounts of spectrin released in the cytosol or buffer during the heat treatment. The amount of spectrin released in the buffer was at least 2.5 times greater than that released in the cytosol, suggesting that the stability of the Triton shells in the presence of cytosol was greater, as compared to that in PBS, during heating.

The observed increased amounts of 70-kDa protein in the membranes of heated erythrocytes cannot be attributed to any physical contamination of the membranes with some 70-kDa protein aggregates that might have formed possibly during heating, as only very little association of this protein was observed with the Triton shells after their incubation with the preheated cytosol at 0 °C for 1 h (Table II). Also, repeated

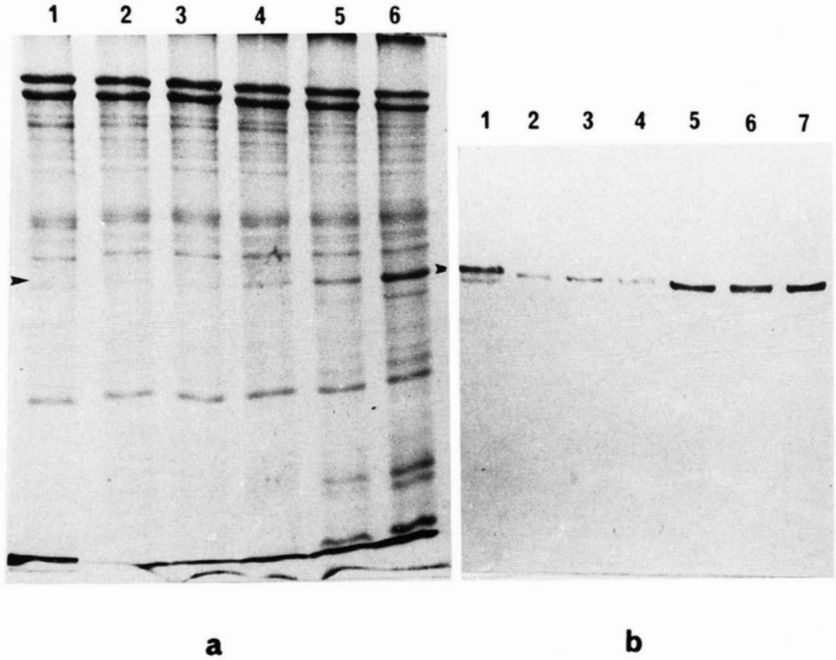

FIG. 3. **Temperature dependence of the cytosol-to-membrane transfer of the 70-kDa protein in rhesus erythrocytes.** The rhesus erythrocytes were incubated at 42, 44, 46, 48, and 50 °C for 15 min. The membranes prepared from these cells were subjected to SDS-PAGE (*panel a*), transferred to nitrocellulose paper, and immunoblotted with hsp 70 mAb and a peroxidase-conjugated secondary antibody (*panel b*). *Panel a: lane 1*, membranes of normal RBC; *lane 2*, membranes of RBC heated at 42 °C; *lane 3*, membranes of RBC heated at 44 °C; *lane 4*, membranes of RBC heated at 46 °C; *lane 5*, membranes of RBC heated at 48 °C; *lane 6*, membranes of RBC heated at 50 °C. *Lanes 2–7* in *panel b* correspond to *lanes 1–6* in *panel a*, while *lane 1* in *panel b* is normal rhesus RBC cytosol. *Arrow* marks the position of the 70-kDa protein in the gels.

washings of the membranes with isotonic saline, both in the presence and absence of EDTA (1 mM), failed to elute the 70-kDa protein from the membranes. Further, the association remained largely unaffected even after treating the membranes with high salt (0.5 M NaCl). Moreover, no change in the intensity of the 70-kDa protein band in the SDS-polyacrylamide gel electrophoretograms was observed by subjecting the cytosol to heating at 44 °C for 1 h (Fig. 6). However, the protein was partially eluted from the membranes together with spectrin-actin, by incubating the heated erythrocyte membranes with low ionic strength buffer (0.1 mM sodium phosphate, 0.1 mM EDTA, 0.2 mM DTT, 1 mM PMSF, pH 8.0) at 4 °C for 36 h or at 37 °C for 30 min.

To examine whether the cytosol-to-membrane transfer of the 70-kDa protein was limited only to the thermally stressed cells, we considered it of interest to look for the presence of this

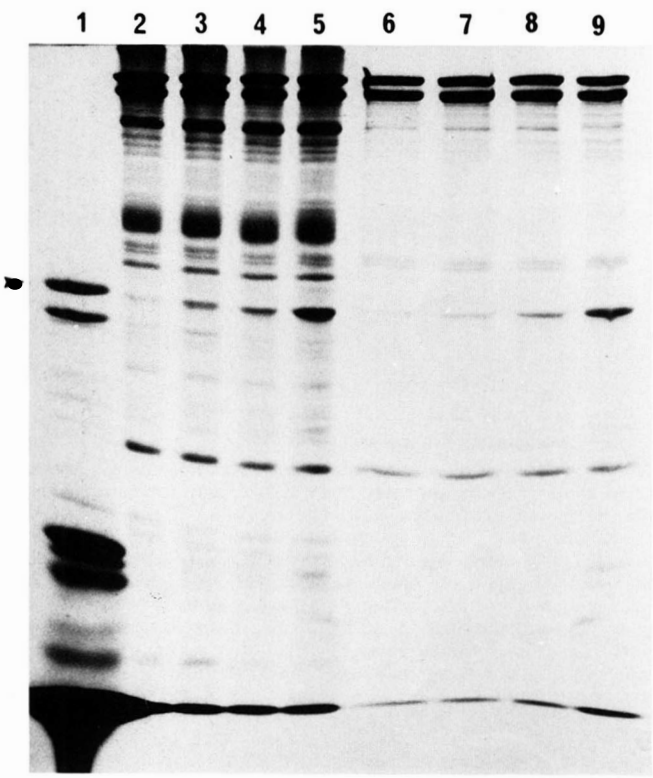

FIG. 4. **Time dependence of the cytosol-to-membrane transfer of the 70-kDa protein in rhesus erythrocytes under heat stress.** After being subjected to heat stress at 44 °C for 1, 2, and 4 h, the rhesus erythrocytes were lysed and membranes were prepared. A portion of these membranes was used in each case to prepare the Triton shells as described under "Experimental Procedures." Both membranes and Triton shells were analyzed by SDS-PAGE. *Lane 1*, normal RBC cytosol; *lanes 2* and *6*, membranes and Triton shells, respectively, derived from normal RBC; *lanes 3* and *7*, membranes and Triton shells, respectively, derived from RBC that were heat-stressed for 1 h; *lanes 4* and *8*, membranes and Triton shells, respectively, derived from RBC that were heat-stressed for 2 h; *lanes 5* and *9*, membranes and Triton shells, respectively, derived from RBC that were heat-stressed for 4 h.

TABLE I
Transfer of 70-kDa cytosolic protein to membrane in rhesus erythrocytes at 44 °C

Erythrocyte treatment	70-kDa protein-to-spectrin ratio in membrane	70-kDa protein-to-spectrin ratio in Triton shells
Normal	0.035	NS[a]
44 °C/1 h	0.095	0.076
44 °C/2 h	0.124	0.099
44 °C/4 h	0.272	0.270

[a] NS, nonsignificant.

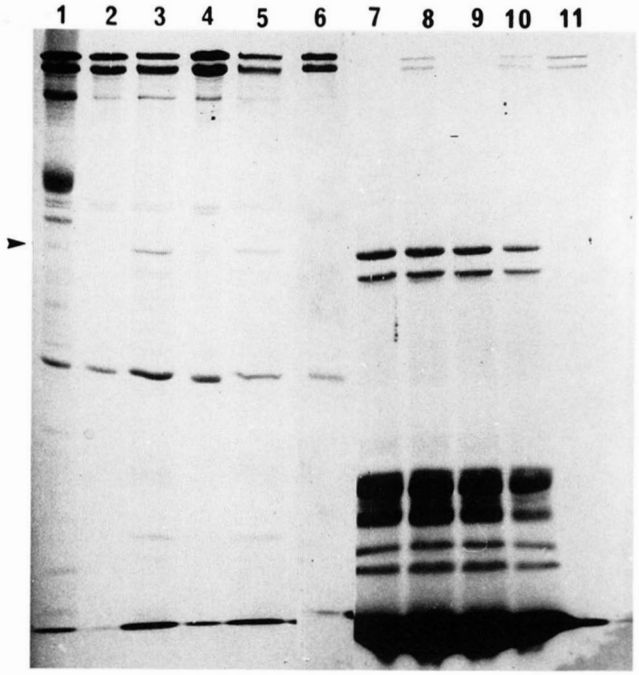

FIG. 5. **Interaction of the rhesus erythrocyte membrane skeleton with the cytosolic 70-kDa protein under heat stress.** Triton shells derived from the normal rhesus erythrocyte membranes were incubated with the cytosol (normal and preheated) and buffer at 0 or 44 °C for 1 h as described under "Experimental Procedures." After incubation, the mixtures were centrifuged, and the pellets and supernatants thus obtained were analyzed by SDS-PAGE. *Lane 1*, rhesus RBC membrane; *lanes 2* and *7*, pellets and supernatants, respectively, obtained after incubating Triton shells with cytosol at 0 °C; *lanes 3* and *8*, pellets and supernatants, respectively, obtained after incubating Triton shells with cytosol at 44 °C; *lanes 4* and *9*, pellets and supernatants, respectively, obtained after incubating Triton shells with preheated cytosol at 0 °C; *lanes 5* and *10*, pellets and supernatants, respectively, obtained after incubating Triton shells with preheated cytosol at 44 °C; *lanes 6* and *11*, pellets and supernatants, respectively, obtained after incubating Triton shells with buffer at 44 °C. *Arrow* marks the position of the 70-kDa protein in the gels.

TABLE II
Interaction of Triton shells with cytosol in vitro

Sample (treatment)	70-kDa protein-to-spectrin ratio[a] in Triton shells
TS[b] + normal cytosol (0 °C/1 h)	NS[b]
TS + cytosol (44 °C/1 h)	0.165
TS + preheated[c] cytosol (0 °C/1 h)	0.048
TS + preheated cytosol (44 °C/1 h)	0.292
TS + buffer (44 °C/1 h)	NS

[a] The ratio was calculated from the densitometric scans of Coomassie Blue-stained SDS-polyacrylamide gel electrophoretograms of the washed pellets obtained after centrifuging the incubation mixtures. The release of spectrin from the Triton shells during heating was never more than 5%.

[b] TS, Triton shells; NS, nonsignificant.

[c] Preheating of cytosol was carried out at 44 °C for 1 h.

protein in the membranes of the erythrocytes that were subjected to stress by the natural conditions. Keeping this in view, we analyzed the membrane protein composition in the rhesus erythrocytes harboring the schizont stage of the simian malarial parasite *P. knowlesi*. Fig. 7 shows that the membranes of these cells did contain good quantities of a 70-kDa protein which strongly cross-reacted with the anti-rhesus erythrocyte 70-kDa cytosolic protein antibodies.

In order to further analyze the nature of this 70-kDa protein, we undertook purification of the protein from the rhesus erythrocyte cytosol. Most of the hemoglobin along with several other undesirable proteins was removed by an ion exchange chroma-

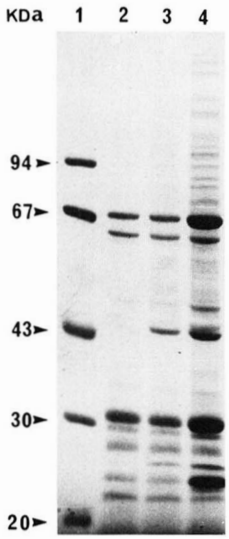

FIG. 6. **Effect of heat on the rhesus erythrocyte cytosolic proteins.** Rhesus RBC cytosol was subjected to heat treatment at 44 °C for 1 h or at 50 °C for 15 min. It was centrifuged at 20,000 × *g* for 30 min. The pelleted proteins in case of the 50 °C-heated cytosol and the lowermost fraction[2] of the 44 °C-heated cytosol were analyzed by SDS-PAGE. *Lane 1*, molecular mass markers; *lane 2*, normal rhesus RBC cytosol; *lane 3*, lowermost portion of the 44 °C-heated cytosol; *lane 4*, pelleted proteins in the case of 50 °C-heated cytosol.

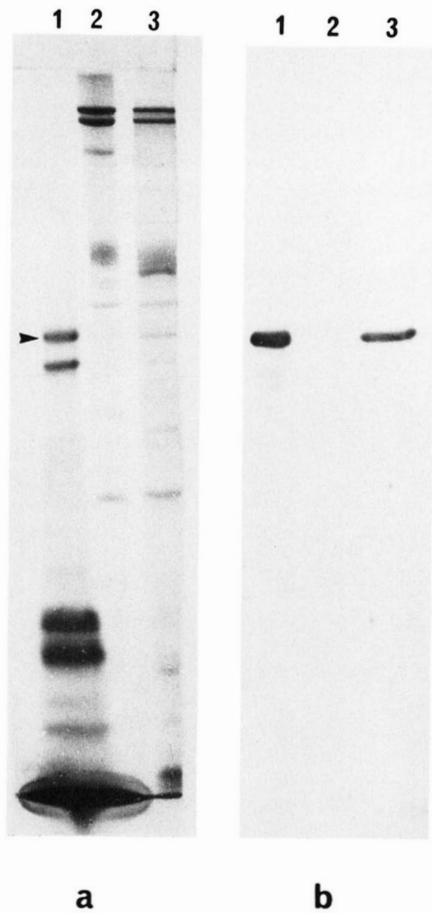

FIG. 7. **Presence of a 70-kDa protein similar to rhesus RBC 70-kDa cytosolic protein in the membranes of rhesus erythrocytes harboring the schizont stage of the simian malarial parasite *P. knowlesi*.** Membranes from normal and *P. knowlesi*-infected rhesus RBC were prepared as described under "Experimental Procedures." These were subjected to SDS-PAGE (*panel a*), transferred to nitrocellulose paper, and immunoblotted with rabbit anti-rhesus RBC 70-kDa cytosolic protein primary antibodies and a peroxidase-conjugated secondary antibody (*panel b*). *Lane 1*, rhesus RBC cytosol; *lane 2*, membranes from normal rhesus RBC; *lane 3*, membranes from *P. knowlesi*-infected rhesus RBC. *Arrow* marks the position of the 70-kDa protein in the gels.

tography of cytosol over DE52. This was followed by an adsorption chromatography on a hydroxyapatite column, which successfully removed almost all the major contaminants from the 70-kDa protein. The remaining impurities were removed either by the HPLC on a preparative gel filtration column or by affinity chromatography over ATP-agarose. The 70-kDa protein on gel filtration eluted primarily under two peaks (Fig. 8*A*). While the first peak, besides containing the 70-kDa protein, also contained some low molecular mass proteins, the second peak was the major peak that contained pure 70-kDa protein. The 70-kDa proteins eluted under the two peaks were different as judged by their cross-reactivity with hsp 70 mAb (Fig. 8*A*, *inset b*). This is further suggested by our finding that in spite of using a large excess of ATP-agarose, only a small fraction of the 70-kDa proteins got bound to the affinity matrix. Interestingly, the fraction that had affinity for ATP-agarose also cross-reacted with hsp 70 mAb (Fig. 9). The isoelectric point of this ATP-binding protein, as determined by the O'Farrell technique (24), was around pH 5.6.

DISCUSSION

This study demonstrates the presence of at least two proteins in the 70-kDa region of the rhesus erythrocyte cytosolic proteins, one (minor component) of which seems to resemble the well known class of hsp 70 (1–6), as judged from its cross-reactivity with hsp 70 mAb, affinity to ATP, and acidic nature (pI ~ 5.6). The second protein, which is also the major protein, neither binds ATP nor does it cross-react with hsp 70 mAb, indicating a marked difference from the hsp 70-like proteins in its properties. That these proteins are different from each other is further suggested by their behavior on the HPLC gel filtration column (Fig. 8*A*); the hsp 70-like protein eluted faster than the major 70-kDa protein. Based on its faster mobility on the gel filtration column, it may be envisaged that the hsp 70-like protein perhaps exists as an oligomer or as a complex with some low molecular weight cytosolic proteins.

These results clearly indicate that the mature rhesus erythrocyte contains an hsp 70-like protein in its cytosol. A similar protein is present also in the mature human red cells, although in reduced quantities. The presence of such proteins in cells of the erythroid origin has earlier been reported by numerous investigators. For example, Singh and Yu (25) have observed an accummulation of hsp 70-like protein in the human erythroid cell line K 562 by inducing its differentiation with hemin. Also, the human reticulocytes and erythrocytes have been shown to possess an hsp 70-like protein which exhibits clathrin uncoating ATPase activity (8). Furthermore, similar proteins have also been identified in erythroid cells of other species (26–28).

The 70-kDa proteins, although localized mainly in the cytosol, could also be detected in the membranes of normal rhesus erythrocytes. The membrane-associated amounts of these proteins increased by subjecting the cells to heat stress, indicating their greater affinity to the membrane under these conditions. As the membrane-associated protein is almost exclusively localized in the membrane skeleton, we suggest that the 70-kDa protein could perhaps preferentially interact with spectrin in the rhesus erythrocytes under thermal stress. This is based primarily on the following considerations: (i) spectrin is known to be the first skeletal protein to undergo unfolding at higher temperatures (11–15), (ii) the hsp 70 family of proteins bind only the partially unfolded protein structures (7), and (iii) the

[2] No protein pellet was formed in this case.

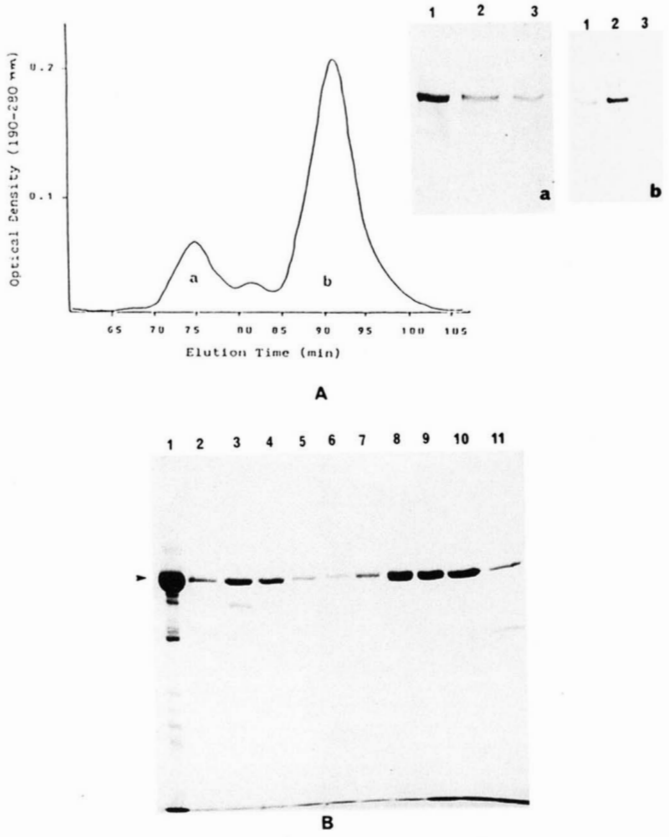

FIG. 8. **High pressure liquid chromatography of the 70-kDa protein-rich fractions, obtained from the hydroxyapatite column, over a TSK-SWG-300 column.** The column was eluted with PBS (*panel A*). The various fractions under the two peaks (*a* and *b*) were analyzed by SDS-PAGE (*panel B*). *Lane 1*, the sample loaded on the column; *lanes 2–6*, protein fractions eluted under the *peak a*; *lanes 7–11*, protein fractions eluted under the *peak b*. The fractions that contained relatively pure 70-kDa protein were pooled together and then subjected to SDS-PAGE (*inset a*), transferred to nitrocellulose paper, and immunoblotted with hsp 70 mAb and a peroxidase-conjugated secondary antibody (*inset b*). *Lane 1*, sample loaded on the HPLC column; *lane 2*, 70-kDa protein-rich fractions under the *peak a*; *lane 3*, 70-kDa protein-rich fractions under the *peak b*.

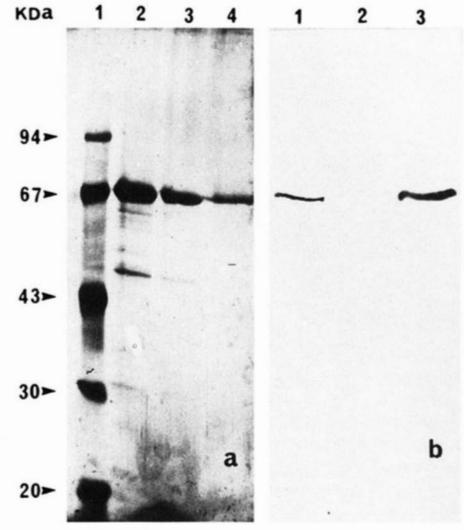

FIG. 9. **Affinity chromatography of the 70-kDa protein-rich fractions obtained from the hydroxyapatite column, over ATP-agarose.** The 70-kDa protein-rich fractions obtained after the hydroxyapatite chromatography were incubated with an excess of ATP-agarose. The unbound proteins were removed, and the bound proteins eluted with 2 mM ATP. The pooled fractions were subjected to SDS-PAGE (*panel a*), transferred to nitrocellulose paper, and immunoblotted with hsp 70 mAb and a peroxidase-conjugated secondary antibody (*panel b*). *Panel a: lane 1*, molecular mass markers; *lane 2*, sample loaded on ATP-agarose; *lane 3*, unbound fractions; *lane 4*, ATP-eluted fractions. *Panel b: lane 1*, sample loaded on ATP-agarose; *lane 2*, unbound fractions; *lane 3*, ATP-eluted fractions.

membrane-associated 70-kDa protein could be extracted only under the spectrin-actin extraction conditions. That hsp 70 can indeed interact with spectrin under stress conditions is further supported by a recent study which has co-localized hsp 70 with spectrin in T-lymphocytes after subjecting them to heat (45 °C, 10 min), phorbol ester, or A23187 treatment (29).

It appears that the heating of the rhesus erythrocytes at 44 °C induces almost selective transfer of a 70-kDa cytosolic protein to the membrane skeleton. Since the membrane-associated 70-kDa protein strongly cross-reacted with both the anti-rhesus erythrocyte 70-kDa protein antibody and hsp 70 mAb, we infer that the membrane-associated protein could primarily represent the hsp 70-like protein present in the erythrocyte cytosol. This suitably explains the present finding that the extent of the cytosol-to-membrane transfer of the 70-kDa protein appeared to increase with temperature when probed by the polyclonal antibody, but it seemed to remain unaltered after 46 °C by probing with hsp 70 mAb.

The association of hsp 70-like protein with membrane skeleton seems to be specific, as this protein was found to be present almost exclusively in the Triton-insoluble rather than the Triton-soluble fractions of the heated rhesus erythrocytes. Also, incubation of the Triton shells with rhesus erythrocyte cytosol at 44 °C resulted in an almost selective transfer of the 70-kDa protein from the cytosol to the skeleton. Further, this protein after binding with membrane skeletons could be eluted only under the spectrin-actin extraction conditions but not by the repeated washings with isotonic saline, 1 mM EDTA, or 0.5 M NaCl. Furthermore, the binding was observed only after heating the cytosol with membrane skeletons at 44 °C but not by incubating the membrane skeletons with preheated (44 °C, 1 h) cytosol at 0 °C, which was quite in agreement with the earlier studies which showed that hsp 70 binds only the partially folded protein structures (7).

The present study shows that the hsp 70-like protein present in the erythrocyte cytosol has a marked tendency to associate with the membrane skeleton under heat stress. This is consistent with the earlier studies which have demonstrated an association between the hsp 70 family of proteins and cytoskeleton under normal and stressed conditions in a variety of cells. Thus, hsp 70 has been found to bind tubulin in heat-shocked 9L rat brain tumor cells (30). Also, its association with microtubules has been demonstrated in mitotic and interphase NIL 8 hamster fibroblasts (31) as well as in rat brain (32). Further, proteins of this family have been reported to co-localize with cytoskeleton in heat-stressed mammary tumor clone C cells (33) and chicken embryo fibroblasts (34).

It would seem that the rhesus erythrocyte hsp 70-like protein binds the membrane skeletal protein(s) in their partially unfolded/denatured form. The binding appears to primarily involve the hydrophobic interactions and is restricted not only to the heat-stressed cells but could also be observed in malaria parasite-infected erythrocytes. Since a similar protein is shown, by us and others (8), to be present also in the mature human erythrocytes, it is likely that the human erythrocyte hsp 70 could also interact with the membrane skeleton under stress conditions. To examine this possibility, we studied the cytosol-to-membrane transfer of this protein in heat-stressed (50 °C, 15 min) human erythrocytes using hsp 70 mAb as the antibody probe. Fig. 10 shows that a protein band strongly reactive to hsp 70 mAb was present in the 70-kDa region of the human erythrocyte membrane proteins. This membrane-asso-

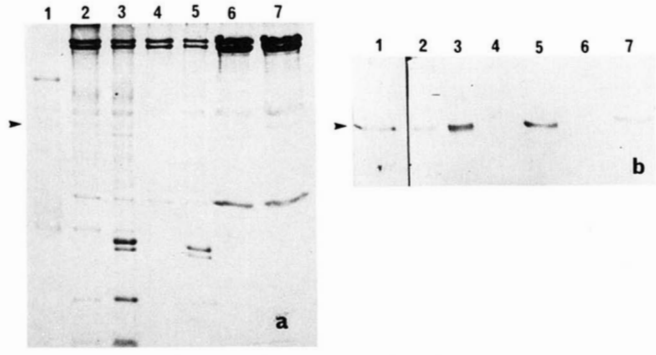

FIG. 10. **Cytosol-to-membrane transfer of the 70-kDa protein in heat-shocked human erythrocytes.** The human erythrocytes were subjected to heat stress at 50 °C for 15 min, and the membranes, Triton shells, and spectrin-actin extract were prepared. These were subjected to SDS-PAGE (*panel a*), transferred to nitrocellulose paper, and immunoblotted with hsp 70 mAb and peroxidase-conjugated secondary antibody (*panel b*). *Lane 1*, RBC cytosol; *lane 2*, normal RBC membrane; *lane 3*, membranes of heat-shocked RBC; *lane 4*, Triton shells derived from normal RBC; *lane 5*, Triton shells derived from heat-shocked RBC; *lane 6*, spectrin-actin extracted from membranes of normal RBC; *lane 7*, spectrin-actin extracted from membranes of heat-shocked RBC.

ciated 70-kDa protein in heated human red cells, like the heated rhesus erythrocytes, was localized mainly in the membrane skeleton and could be partially extracted from the membranes under the spectrin-actin extraction conditions.

Mature mammalian erythrocytes are not capable of synthesizing any new proteins and, therefore, the hsp 70-like protein(s) seen in these cells should have been made at some early stage during differentiation, such as at the reticulocyte stage (8, 28). These proteins thus closely resemble the hsc 70 class of proteins which are constitutively expressed in a variety of normal cells and are primarily cytoplasmic (1–3). The major function which these proteins might perhaps perform in the mature erythrocytes is to provide protection to these cells under stress conditions, such as metabolic starvation, fever, etc., by binding with the partially damaged skeletal protein structures. That this binding may possibly protect the cells against further stress-induced structural damage is suggested by our finding that the release of free spectrin from the membrane skeletons is increased at least 2.5 times by their heating in buffer rather than in the cytosol. This suggestion finds further support from the earlier studies which have shown that association of hsp 70 with cytoskeleton renders the cells resistant to further stress-induced structural damage (30, 33, 34).

In summary, the mature rhesus erythrocytes contain significant quantities of a cytosolic protein which closely resembles the hsp 70 class of proteins. This protein under normal conditions is localized mainly in the cytosol, but, under stress conditions (*e.g.* high temperature, malarial infection, etc.), it has a tendency to associate with the membrane skeleton. The association is primarily through the hydrophobic interactions and perhaps provides protection to the membrane skeleton against the stress-induced structural damage. Based on these observations, we conclude that the hsp 70-like proteins in the mature mammalian erythrocytes could possibly play an important role in protecting the cells under stress conditions by stabilizing the membrane skeleton through their association with skeletal proteins.

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