T-complex Polypeptide-1 Interacts with the Erythrocyte Cytoskeleton in Response to Elevated Temperatures

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Chaperonins are double ring complexes composed of highly conserved 60-kDa protein subunits that are divided into two subgroups. Group II chaperonins are found in archaea and the cytoplasm of eukarya and are believed to function like other chaperonins as part of a protein folding system. We report here that human erythrocytes contain the group II chaperonin T-complex polypeptide 1 (TCP-1) and that this complex translocates from the cytoplasm to the cytoskeleton in response to heat treatment in the absence of overt cell damage. Identification as TCP-1 was determined by immunodetection for TCP-1α and corroborated by mass spectrometry peptide sequencing. Direct visualization by immunofluorescence confirmed peripherally localized TCP-1 in response to heat treatment. Temperatures ranging from 37–50 °C were demonstrated to have distinct kinetic profiles of induced translocation. Heat-induced binding was shown by Triton shell analysis to be specifically associated with the cytoskeletal proteins. Furthermore, the binding was reversible following removal of the stimulatory condition. A stabilizing process is hypothesized based on the known interactions of chaperonins.

Most cell types have been shown to have a specific and reproducible response to hyperthermic conditions. Elevated temperature-induced chromosomal puffing were the first observed heat shock responses (1) and were later determined to represent transcriptional activation of a select group of proteins (2). Proteins that are characterized by increased production in response to elevated temperatures became known as heat shock proteins (HSPs).1 Subsequent research has shown that these proteins may be regulated by many factors, including environmental stress (heat shock, heavy metals, chemicals), pathophysiologic status (fever, inflammation, ischemia), and certain non-stress conditions (growth, differentiation) (3).

In light of evidence that the major HSPs interact with other proteins, they have been classified as molecular chaperones (4–6).

1 The HSP-60 family, commonly known as chaperonin to reflect their central role in the process of protein folding (6, 7), are double ring structures composed of multiple 60-kDa subunits and are subdivided into two groups based on sequence and structural information (8). Group I chaperonins are formed as double rings of identical or closely related protein subunits with seven subunits per ring and include HSP-60 from bacteria, mitochondria, and chloroplasts. Group II chaperonins are formed as double rings of identical or slightly different, but homologous, protein subunits with eight or nine subunits per ring depending on the species. This group of chaperonins includes thermosomes and rosettasomes in archaea and TCP-1 in eukarya. Previous reports demonstrate that in archaea group II chaperonins are composed of heat shock proteins (9), whereas the eukaryotic TCP-1 is composed of 60-kDa proteins that are not heat stress-inducible (10). Both archaeal and eukaryal group II chaperonins are isolated from cells as double ring structures, although it has been observed that at least archaeal chaperonins spontaneously aggregate into filaments (11), which has led to the suggestion that they may play a structural role in vivo (12). In addition, other functions have been suggested for group II chaperonins, including RNA (13) and membrane stabilization (14).

Our laboratory has been studying novel stabilization mechanisms for erythrocyte storage and recently reported a process for enhanced stability and recovery of cells following cryopreservation (15). We began investigating temperature-induced changes to erythrocyte protein and membrane organization as a consequence of those studies. Mammalian erythrocytes serve as an ideal model system to investigate mechanisms of membrane/cytoskeletal stability and inducible changes therein because mature erythrocytes lack a nucleus and do not synthesize new proteins. Thermotolerance studies of erythrocytes have previously been reported with mixed results. Studies in mammalian erythrocyte systems have shown little or no inducible thermotolerance as detected by osmotic fragility and leakage/hemolysis indices (16–18). The degree to which hemolysis occurs as a function of incubation temperature and time is also not firmly established (17, 19, 20). Changes in cytoskeletal proteins, principally spectrin extractability and dimer/tetramer ratio, have been reported following incubation (21–23). Although functional thermotolerance remains to be firmly established, small quantities of new proteins, presumably of cytoplasmic origin, have been observed in membrane preparations following heating (21, 22), including HSP-70 (24). In addition to HSP-70, HSP-90 has been identified in mammalian erythrocytes (25), although their functional roles are not known. These changes may help account for reported alterations in biorheological properties following heat treatment (23).
TCP-1 Interaction with Erythrocyte Cytoskeletons

We report here that human erythrocytes contain TCP-1 and that this cytoplasmic protein complex translocates to the membrane following heat treatment. This heat-induced membrane interaction is consistent with recent findings that that the archaean group II chaperonins in Sulfolobus shibatae are also membrane-associated (26), suggesting the possibility that a membrane-related function for group II chaperonins is widely distributed and evolutionarily conserved.

EXPERIMENTAL PROCEDURES

Erythrocyte Handling—Packed erythrocyte units were purchased (Gulf Coast Regional Blood Center, Houston, TX) following standard quality control testing. The units were maintained at 4 °C at all times and used within 10 days following donation. In some experiments, erythrocytes were prepared by standard centrifugation procedures (27) from whole blood freshly drawn into acid citrate dextrose anticoagulant following donor consent. All freshly prepared erythrocytes were used within 6 h.

Prior to experimental work, the erythrocytes were washed with a buffer solution (50 mM sodium chloride, 110 mM glucose, 10 mM glutamine, 55 mM mannitol, 2 mM adenosine, pH 6.1, 280 mosM) (described in Ref. 28) and adjusted to a working hematocrit of 30%. Heat treatment was performed in a water bath at temperatures from 37–55 °C. Small volumes (less than 5 ml) in polypropylene tubes were used to ensure rapid and uniform temperature changes within the erythrocyte sample.

Hemolysis Determination—Cell suspensions were analyzed for erythrocyte number, hematocrit, and total hemoglobin using a Biochem Immunosystems 1099+ hematolysis analyzer. Free hemoglobin concentrations were determined spectrophotometrically using a quantitation kit (Sigma) on a cell-free sample isolated by centrifugation. Hemolysis was determined as the ratio of free hemoglobin to total hemoglobin normalized by hematocrit, as described elsewhere (29).

Erythrocyte Protein Preparation—Membrane/cytoskeletal and cytoplasmic proteins were prepared by the formation and isolation of erythrocyte ghosts. Ghosts were formed by hypotonically lysing 1 ml of erythrocyte sample in 30 ml of ice-cold phosphate buffer (5P8; 5 mM sodium phosphate, 0.5 mM EGTA, 20 μg/ml phenylmethane-sulfonyl fluoride, pH 8.0). Following centrifugation at 30,000 × g for 4 °C for 15 min, the supernatant (30 ml), containing cytoplasmic proteins, was recovered and saved; the pellet, containing erythrocyte ghosts, was washed three times with fresh ice-cold 5P8 until it was free of hemoglobin. The volume of the ghost sample was adjusted to 1 ml with 5P8. These ghosts were also used to prepare “Triton shells” in some experiments by incubating for 30 min at 4 °C in 20 volumes of Triton buffer (625 mM NaCl, 625 mM sodium phosphate, 0.625 mM EGTA, 0.625 mM dithiothreitol, and 2% Triton X-100), centrifuging as above, washing in ice-cold 5P8 to remove residual Triton buffer, and resuspending in fresh, ice-cold 5P8. 0.1% (w/v) sodium dodecyl sulfate (SDS) was added to ghost and Triton shell samples, and total protein was determined using a bicinchoninic acid protein quantification kit (Pierce). Total protein in the cytoplasmic fraction was not quantified because of the high concentration of hemoglobin. Alternatively, a ratio method was used to determine the appropriate volume for analysis and is described in detail below.

Electrophoretic Protein Separation—Protein samples were separated by SDS-PAGE using an 11.5% resolving gel. Typically, a membrane aliquot containing 30 μg of membrane protein was loaded per lane. However, analysis of the cytoplasmic fraction was complicated by both the high concentration of hemoglobin and the large volume generated during hypotonic lysis. To allow for quantitative comparative analysis, the volume of the membrane protein aliquot was used to calculate the volume of a cytoplasmic protein aliquot representing one-fourth the number of cells using a volumetric ratio method as shown in Equation 1.

\[
\text{cytoplasmic aliquot volume} = \frac{\text{membrane aliquot volume} \times 30 \text{ ml}}{0.25} \quad (\text{Eq. 1})
\]

The cytoplasmic aliquot was dried for 5 h in a Savant SC110A SpeedVac concentrator at ambient temperature, and the resulting pellet was resuspended in standard gel loading buffer. These membrane and cytoplasmic protein samples represent known numbers of cells and were analyzed side-by-side by SDS-PAGE. The resulting gel was either stained with Coomassie Blue (Bio-Rad) or blotted to polyvinylidene difluoride membrane for Western blot analysis. The one-fourth cell number value was used after initial attempts to analyze equivalent cell number aliquots failed due to excessive protein in the cytoplasmic samples fouling the gel.

Western Blot Analysis—Proteins of interest were detected by indirect enhanced chemiluminescence (ECL) using ECL-Plus (Amersham Biosciences). Blots containing samples of cytoplasmic proteins were washed with 3% hydrogen peroxide to oxidize hemoglobin prior to primary antibody incubation, thereby preventing a nonspecific luminal reaction during ECL detection. Primary antibodies against human TCP-1α, TCP-1β, and HSP-60 (Stressgen, Victoria, BC, Canada) were used at 1:3,000 dilution. Species-appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma) were used at dilutions between 1:50,000 and 1:80,000. ECL signals were detected on Hyperfilm (Amersham Biosciences), which were then analyzed by densitometry using Quantity One software (Bio-Rad).

Immunofluorescence Analysis—100 μl of the erythrocyte suspension was mixed with 1 ml of isotonic 2.5% low melting point agarose, poured onto paraffin, and allowed to solidify. A small piece of the solid agarose was wrapped in histology processing paper, placed in a processing cassette, and dehydrated by serial incubation in 70, 80, 90, and 100% (twice) ethanol for 30 min each. The dehydrated sample was cleared twice in chloroform for 40 min each, embedded in paraffin, sectioned (~4-μm thick), placed on a glass slide, deparaffinized with chloroform for 20 s, and rinsed in deionized water. Slides were blocked with 5% rabbit serum (host species of secondary antibody), incubated with a 1:500 dilution of primary anti-TCP-1α for 2 h, and finally with a 1:5,000 dilution of fluorescein isothiocyanate-anti-rat IgG (Sigma) for 2 h. Fluorescent images were digitally captured from a fluorescent microscope.

RESULTS

The interaction of TCP-1α with the erythrocyte membrane/cytoskeleton was observed in response to heat treatment. Immunodetection of TCP-1α in the membrane fraction revealed a significant positive response in heat-treated cells that was absent in non-heat-treated controls (Fig. 1). A sample of the treated erythrocytes was evaluated for hemolysis and yielded less than 1% free hemoglobin, indicating that the temperature incubation did not induce hemoglobin leakage or cell lysis. Membrane-bound TCP-1α in response to heat treatment is cytoplasmic in origin. Cytoplasmic protein analysis demonstrated a strong detection in both control and heat-treated erythrocytes using the described modified Western blotting technique (Fig. 1). Adjusting for the one-fourth cell number ratio and normalizing to cytoplasmic control levels, densitometry analysis revealed a significant reduction in detected band intensity following heat treatment to 72.3% for the figure shown and 69.8% ± 10.2% for all analyses. Similar analysis of heat-treated membrane intensity yielded 19.6% for the figure shown and 19.2% ± 4.3% for all analyses. The heat-treated membrane and cytoplasmic intensities additively account for nearly all of the cytoplasmic control intensity, 91.9% in the figure shown and 89.0% ± 6.8% overall. Finally, erythrocytes made into ghosts prior to heat treatment do not demonstrate a positive TCP-1α signal by Western blotting, whereas the same unit of erythrocytes responded to heat treatment as shown in Fig. 1 (not shown).

The results above rely on immunodetection for identification of TCP-1α. Definitive identification was performed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry protein sequencing (Protein Chemistry Core Facility, Columbia University, New York, NY) on an immuno-positive spot selected from a two-dimensional SDS-PAGE gel (Kendrick Laboratories, Madison, WI). MALDI analysis was performed blind and confirmed identification as TCP-1α. Furthermore, Western blot analysis of membrane-associated proteins from heat-treated erythrocytes was positive for TCP-1β, and the spatial relationship between TCP-1α and -1β on two-dimensional blots corresponded to previously published results (30) (see supplemental online material for two-dimensional analysis and additional information).

Translocation of TCP-1α from the cytoplasm to the mem-
brane of erythrocytes was found to be both temperature- and time-sensitive. Fig. 2 shows cumulative results of this temperature-time profile. Temperatures greater than 45 °C induced a rapid translocation event and achieved maximal levels after 30 min of incubation. Erythrocytes incubated at 37 and 40 °C showed a slower onset of translocation but did yield evidence of translocation within 3 h of incubation. Despite the difference in rates observed between temperatures, erythrocytes incubated at all temperatures tested eventually displayed a significant level of translocation as compared with background. Free hemoglobin was measured relative to total hemoglobin to assess potential damage. In all cases, the level of free hemoglobin was less than 1%, indicating no hemoglobin leakage or cell lysis during treatment.

The results described above establish a heat-induced translocation of TCP-1α from the erythrocyte cytoplasm to the membrane but depend on adequate separation of cytoplasmic and membrane proteins to distinguish between these two protein reservoirs. An immunofluorescent histological technique was used to demonstrate the translocation event without separating the two protein fractions. Fluorescent detection revealed a diffuse pattern of uniform intensity consistent with general cytoplasmic localization in the control erythrocytes (Fig. 3C). Upon heat treatment, the erythrocytes displayed a pattern of fluorescence observed as a defined ring structure associated with the periphery of the cell (Fig. 3D). Furthermore, the heat-treated cells retained a significant level of diffuse uniform cytoplasmic signal, which is consistent with the cytoplasmic results shown in Fig. 1.

The translocation of TCP-1α to the membrane protein fraction of heat-treated erythrocytes does not reveal the nature of the interaction with the erythrocyte membrane fraction. To determine this interaction, Triton shells, a subcomponent of erythrocyte ghosts that contains primarily the cytoskeletal proteins, were generated from control and heat-treated erythrocyte ghosts. Total protein from intact ghosts and Triton shells was analyzed by Western blot. Representative results of this experimental series, shown in Fig. 4, demonstrate that the translocated TCP-1α is detectable in both the complete ghost membrane (G) and Triton shell (T) proteins. These data indicate that the translocated TCP-1 complex is specifically linked to the erythrocyte cytoskeleton.

Although no hemoglobin leakage or gross hemolysis was observed in response to heat treatment, irreversible translocation of TCP-1α would indicate a low level of damage that may precede other forms of erythrocyte damage. A reversibility experiment was performed to test the persistence of TCP-1α translocation. Heat-treated and untreated control samples were transferred to 37 °C, and membrane proteins were isolated from these samples at various time points. Fig. 5 shows that heat-treated erythrocytes have a significant positive TCP-1α signal that declines in intensity to background levels over the 30 h of this experiment. The control cells showed an increase in intensity during the first 6 h of incubation and a decline, although not complete, during the remainder of the experimental time frame. The pattern observed in the control cells is consistent with the translocation kinetics displayed in Fig. 2. Despite the induced translocation at 37 °C (this response will be addressed under “Discussion”), both control and heat-treated erythrocytes exhibited a reversal of TCP-1α translocation. This reversal was not observed when the control and treated cells were transferred to 4 °C rather than 37 °C (not shown).

All of the experimentation reported above was performed using erythrocytes processed according to approved blood bank procedures. Heat treatment-induced translocation was tested in freshly prepared erythrocytes to ensure that these procedures do not influence TCP-1α translocation. Fig. 6 shows total membrane protein analysis and Western blot detection of TCP-1α for untreated and heat-treated erythrocytes from three different donors. Despite slight differences in background levels, all three donors exhibited a strong translocation event in

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**Fig. 1. Western blot analysis of erythrocyte TCP-1α.** Erythrocytes were left untreated as a control (C) or heat-treated at 48 °C for 20 min (H) and then hypotonically lysed. The membrane and cytoplasmic protein fractions were isolated. Using the control membrane fraction as a reference, total membrane protein from an equivalent number of cells and total cytoplasmic protein from one-fourth the number of cells were referenced to the total amount of TCP-1α in the control cytoplasm after adjusting for the dilution ratio. This figure is representative of analysis on three separate donors.

**Fig. 2. Relative quantization of TCP-1α translocation.** Erythrocytes were heated for increasing lengths of time at the specified temperature. Total membrane protein was isolated, and equal amounts were separated by SDS-PAGE. The gel was blotted and analyzed for TCP-1α by Western detection. The blots were scanned, and band intensity was determined by densitometry. Within a single experiment, the intensities were normalized to 50 °C for 30 min. The values represent mean ± S.D. for n = 3 separate donors.
DISCUSSION

The TCP-1 complex has previously been isolated from reticulocytes, a circulating nucleated precursor of mature erythrocytes (34), but this report represents the first identification of TCP-1 in the cytosol of human erythrocytes. The dearth of information with respect to heat shock proteins and erythrocytes stems from two factors unique to these cells. First, the prevailing view of erythrocytes is one of a circulating cell passively distributing oxygen, carbon dioxide, and other labile gases based solely on mass transport properties and lacking in much of the cellular biochemistry associated with nucleated cells. Secondly, human erythrocytes lack nuclei and other internal organelles and thus have no mechanism for de novo protein synthesis in response to cellular insult. Because the understood mechanism of the heat shock response involves the synthesis of nascent proteins, erythrocytes by definition cannot have a classical heat shock response. The presence of other heat shock proteins in human erythrocytes, including HSP-70 and HSP-90, has been reported (25); however, the exact function of these proteins has not yet been defined. Results from erythrocytes of non-human species have led to stabilization hypotheses (21, 35). In particular, an HSP-70-like protein in Rhesus erythrocyte cytoplasm was shown to translocate to the cytoskeleton at 44 °C, whereas a temperature of 50 °C was necessary to induce translocation of this protein in human erythrocytes (21). However, the high temperature necessary to induce detectable levels of HSP-70 negates any physiologic relevance.

Chaperonins are generally believed to be localized to the cytoplasm. TCP-1 is included in the 60-kDa subunit double ring chaperonin family despite a relatively short, albeit growing (reviewed in Ref. 5), list of reported protein interactions. There is further evidence that members of both Group I (36–39) and Group II (40–42) chaperonins are associated with the membrane. However, the membrane associations of the Group II TCP-1 chaperonin all occur under native, non-stressed conditions, and in mammalian systems the association has only been observed on membranes of internal organelles (40, 42). TCP-1 is generally not considered to be a heat shock protein, although one report briefly mentions inducibility in human cultured cells (43). We found no previous reports of thermal-induced TCP-1 association with membranes as we have observed for human erythrocytes.
TCP-1 Interaction with Erythrocyte Cytoskeletons

Our results with human erythrocytes demonstrate translocation of TCP-1, as detected by TCP-1α, from the cytoplasm to the membrane in response to a range of elevated temperatures. 20–50% of the total TCP-1 reservoir translocates from the cytoplasm to the membrane (Fig. 1). Approximately 90% of the original cytoplasmic TCP-1 is accounted for by performing an additive analysis of the heat-treated membrane and cytoplasmic fractions. The number of manipulations and semi-quantitative nature of the experimental methods preclude achieving a 100% additive level. The extremely high protein concentrations associated with the cytoplasmic fraction contributed to the experimental and analytical complexities, such as the lane spreading observed in Fig. 1. Despite these issues, the cytoplasmic reservoir of TCP-1 clearly exceeds the amount of translocation. This finding is consistent with the concept of a fast-acting response mechanism, particularly in cells lacking de novo protein synthesis capabilities.

The complex primarily associates with the membrane skeleton based on Triton shell analysis. The proteins in the Triton shell include spectrin, actin, and junctional proteins, any of which could be TCP-1 binding sites. Among cytoskeletal proteins, TCP-1 has been shown to interact with and structurally fold actin and tubulin (44–46), and mutants in yeast contain cytoskeletal defects characteristic of malfunctioning actin and tubulin (47). Because erythrocytes lack tubulin and other microtubule structures but do contain short actin filaments at crucial cytoskeletal junctions that remain with the Triton shell, actin represents a strong candidate protein for the specific interaction.

Physiologic relevance is provided through the combination of two specific results. First, the translocation event was observed at temperatures that fall within the physiologically relevant range (Fig. 2). Second, TCP-1 is released from the membrane when returned to physiologic temperatures in an absence of hemolysis (Fig. 5). The data generated at 40 °C, which represents an achievable fever environment, clearly yielded significant translocation of TCP-1 to the cytoskeleton. The kinetic profile of translocation for heat incubation was also performed at 42 °C. The profile of this temperature was extremely donor-dependent and seemed to fall into a transitional zone between slow (temperatures <42 °C) and rapid (temperatures >42 °C) translocation. This profile was not included in Fig. 2 because of the variability.

The ability of cells to achieve thermotolerance, stability at lethal temperatures as a result of prior conditioning at an elevated sub-lethal temperature, correlates well with production of HSPs (48, 49). Despite studies supporting the participation of HSPs in acquired thermotolerance (50–55), the exact set of HSPs necessary remains elusive (56–60). The ability of erythrocytes to achieve thermotolerance has yet to be firmly established in published literature. Classic indices of erythrocyte damage generally indicate a loss of functional capabilities in conjunction with any detectable heat-induced protein changes. Koter and Laski (17) demonstrated a level of thermotolerance following preconditioning at 44 °C. However, the thermotolerant cells were significantly damaged compared with non-treated controls with respect to osmotic fragility and hemolysis. Key biorheological properties are also diminished following heating of erythrocytes (23, 61). These biorheological changes may be due to alterations in the cytoskeletal structure, which is demonstrated by decreased spectrin-actin extraction and an increased spectrin dimer-to-tetramer ratio following heat treatment (21, 22). The majority of these studies were performed at temperatures greater than 42 °C and, therefore, may not represent physiologically relevant events.

The work presented here is primarily based on erythrocytes derived from blood banking procedures where the packed cells are stored at 4 °C according to approved methods for transfusion medicine. An analysis of freshly isolated erythrocytes, cells that were never exposed to temperatures less than 37 °C prior to experimentation, exhibited the same translocation event in response to heat treatment. This result in part validates the use of blood bank erythrocytes but does not establish that the blood banking process of storing erythrocytes at 4 °C is non-damaging. In fact, the observed response of blood bank cells to incubation at 37 °C indicates some alteration in the nature of the cells (Fig. 5B). Because the freshly isolated erythrocytes were maintained at 37 °C for the entire duration of the experiment, the resulting control samples, those that never were exposed to higher temperatures, provide insight into basal levels of TCP-1 binding. Despite some degree of donor variability, these control erythrocyte ghost membranes demonstrated that the natural level of bound TCP-1 is extremely low. Thus, the 37 °C results with blood bank erythrocytes showing an induced translocation and reversal are consistent with some level of prior alteration, possibly caused by the initial transfer and storage at 4 °C that has recently been shown to induce a similar TCP-1 translocation (62).

Previous studies showed heat-induced translocation of unknown low molecular weight proteins (21, 22) that, based on the identification here and previously (15), are now known to be carbonic anhydrase and flavin reductase. These enzymes are components of the erythrocyte catalytic machinery catalyzing the hydration of carbon dioxide (carbonic anhydrase) and the reduction of biliverdin to bilirubin in the heme degradation pathway (flavin reductase). The critical functions of these enzymes in toxic waste management may provide insight to their membrane-bound role in response to thermal stress. However, whether translocation of these enzymes or TCP-1 interaction with the erythrocyte cytoskeleton confers a level thermostolerance at physiologically relevant temperatures remain important questions for investigation.

The translocation of TCP-1 to the human erythrocyte cytoskeleton is similar to observations in the archaeal organism _S. shibatae_. This extreme thermophile produces abundant 60-kDa HSPs in response to heat shock that form a rosettasome structure homologous to TCP-1. The newly synthesized protein localizes preferentially to the membrane where it exhibits a protective role in cell stabilization (26). The degree of sequence and structural similarity between the archaeal rosettasome and mammalian TCP-1 leads one to consider an evolutionarily conserved stabilizing mechanism in response to cellular insult.

The results presented in this study demonstrate a heat-induced translocation event of the cytosolic-based TCP-1 complex to the cytoskeleton in human erythrocytes. Previously, TCP-1 was not considered to be a heat shock protein, based on genetic analysis. However, the results presented here with respect to the heat-induced response should qualify TCP-1 as a heat shock protein. This response was observed to occur at temperatures within physiologically relevant ranges and to be reversible following recovery at 37 °C. Although a functional consequence of translocation is not yet known, these results are consistent with a stabilizing mechanism during the period of stress. Because of the relative simplicity of the erythrocyte compared with nucleated cells, this heat-induced translocation may serve as a model to investigate novel TCP-1 functions.

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