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ORIGINAL ARTICLES

Identification of Human Erythrocyte Cytosolic Proteins Associated with Plasma Membrane During Thermal Stress

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Abstract The influence of thermal stress on the association between human erythrocyte membranes and cytosolic proteins was studied by exposing erythrocyte suspensions and whole blood to different elevated temperatures. Membranes and cytosolic proteins from unheated and heat-stressed erythrocytes were analyzed by electrophoresis, followed by mass spectrometric identification. Four major (carbonic anhydrase I, carbonic anhydrase II, peroxiredoxin VI, flavin reductase) and some minor (heat shock protein 90α, heat shock protein 70, α-enolase, peptidylprolyl *cis–trans* isomerase A) cytosolic proteins were found to be associated with the erythrocyte membrane in response to in vitro thermal stress. Unlike the above proteins, catalase and peroxiredoxin II were associated with membranes from unheated erythrocytes, and their content increased in the membrane following heat stress. The heat-induced association of cytosolic proteins was restricted to the Triton shells (membrane skeleton/cytoskeleton). Similar results were observed when Triton shells derived from unheated erythrocyte membranes were incubated with an unheated erythrocyte cytosolic fraction at elevated temperatures. This is a first report on the association of cytosolic catalase, α-enolase, peroxiredoxin VI, peroxiredoxin II and peptidylprolyl *cis–trans* isomerase A to the membrane or membrane skeleton of erythrocytes under heat stress. From these results, it is concluded that specific cytosolic proteins are translocated to the membrane in human erythrocytes exposed to heat stress and they may play a novel role as erythrocyte membrane protectors under stress by stabilizing the membrane skeleton through their interactions with skeletal proteins.

Keywords Erythrocyte · Membrane skeleton · Heat stress · Electrophoresis · Mass spectrometry

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Introduction

A great deal of information is available about the cellular and molecular responses of nucleated cells to hyperthermia, but there are very few reports concerning the effects of heat stress on mammalian erythrocytes, which lack a nucleus and do not synthesize new proteins. Their intracellular environment consists of hundreds of cytosolic proteins including hemoglobin (Hb) and several enzymes. The erythrocyte membrane is made up of a lipid bilayer and a membrane skeleton. The membrane skeleton is composed of spectrin, band 4.1, actin and several minor proteins (Bennett 1990). When erythrocyte membranes are extracted with Triton X-100, the lipids and other proteins like band 3, band 4.5 and band 6 are extracted, leaving behind the "membrane skeleton." The major function of erythrocytes is oxygen transportation, and as a consequence, reactive oxygen species are formed, which could lead to compromised integrity of membranes, cellular lysis, protein modification, lipid peroxidation and Hb denaturation (Halliwell and

Gutteridge 1986; Lii and Hung 1997; Amer et al. 2008; Devasena et al. 2001).

The effects of heat stress on human erythrocyte morphology are well studied, including changes such as budding, fragmentation and transformation to a spheroechinocytic shape (Utoh et al. 1992). One of the methods of investigating damage to the erythrocyte membrane is osmotic fragility. Increased osmotic fragility and hemolysis have been attributed to membrane proteins (Ho and Lin 1991; Jozwiak et al. 1991). A direct correlation between hemolysis and membrane protein denaturation has been reported (Lepock et al. 1989). Heat-induced denaturation of spectrin showed modification in membrane physiochemical properties (Minetti et al. 1986). The spectrin dimer (αβ) undergoes dissociation to form monomers at temperatures of 49–50 °C, followed by aggregation of denatured β-subunits (Yoshino and Minari 1987). The changes in erythrocyte spectrin (dimer to tetramer ratio and extractability) due to heat stress (Yoshino and Minari 1987; Kumar et al. 1990; Gudi et al. 1990) and the role of spectrin thiols in maintaining erythrocyte membrane thermal stability have been reported (Streichman et al. 1988). Ozturk and Gumuslu (2004) studied the age-related changes in antioxidant enzymes and lipid peroxidation in rat erythrocytes after heat stress. Houston and Mearow (1979) reported an increase in erythrocyte carbonic anhydrase (CA) activity of fishes in response to changes in temperature. Alterations in membrane proteins are also reported during oxidative stress, hypoxia and long-term storage of blood (D'Amici et al. 2007; Kriebardis et al. 2007; Rocha et al. 2009). Heat shock proteins (HSP27 and HSP70) and peroxiredoxin II (Prdx II) perform the function of molecular chaperones in mouse erythrocytes during disease conditions (Biondani et al. 2008). HSP70-like protein in rhesus erythrocytes is reported to translocate to the membrane from the cytosol under heat stress, and the same protein in lesser amount is also found in human erythrocytes (Gudi and Gupta 1993). Wagner et al. (2004) have shown T-complex polypeptide 1 (TCP 1) interaction with the membrane when human erythrocyte suspension is exposed to heat at 48 °C. They have also reported the translocation of CA and flavin reductase (FR) from the cytosol to the membrane. There are no reports so far on the effect of heat stress on the association of other erythrocyte cytosolic proteins to the membrane.

This study examined the effect of heat denaturation of a major membrane skeletal protein, spectrin (at 50 °C), on intact erythrocytes. On the basis of the results, we report that exposure of erythrocytes to high temperatures leads to the association of several cytosolic proteins—HSP90α, HSP70, catalase, α-enolase, CA (isoforms I and II), Prdx VI, FR, Prdx II and peptidylprolyl *cis–trans* isomerase A (PPIA)—with the membrane. In addition, this study shows the specific association of these cytosolic proteins to the Triton-generated membrane skeleton.

Materials and Methods

Chemicals

Trifluoroacetic acid, α-cyano-4-hydroxycinnamic acid (CHCA) matrix and calibration standards were obtained from Applied Biosystems (Foster City, CA). All other biochemicals used were of the grade suitable for gel electrophoresis and mass spectrometric analysis.

Preparation of Erythrocytes

Human blood, obtained from healthy volunteers, was collected in anticoagulant tubes containing acid citrate dextrose. Informed written consent was obtained from the voluntary donors. Erythrocytes were obtained by removing plasma and the buffy coat from blood by centrifugation at 1,000×*g* for 5 min at room temperature (RT) and washing four times with 10 volumes of Tris-buffered saline (TBS; 10 mM Tris–HCl [pH 7.4] with 150 mM NaCl) at the same *g* value.

Heat Treatment and Measurement of Osmotic Fragility

Aliquots of whole blood and erythrocyte suspensions (ES; 45 % hematocrit, $n = 4$) were heated for exactly 15 min at different elevated temperatures—44, 46, 48 and 50 °C—in a serological water bath. The tubes were cooled to room temperature. Whole blood was centrifuged at 1,000 x *g* for 10 min to pellet the erythrocytes, which were then washed with TBS. The osmotic fragility of the unheated and heated erythrocyte suspensions (UnHEs and HEs) was measured essentially according to Jaja et al. (2002).

Lysate and Membrane Preparations

Membranes were prepared from UnHEs and HEs ($n = 10$) according to Hanahan and Ekholm (1974) with the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). In the last washing, 0.05 % NaN_3 was added to the lysis buffer (10 mM Tris–HCl, pH 7.4) to prevent microbial growth. The membrane preparations so obtained are labeled UnHEMs or HEMs. UnHEs and HEs were lysed by mixing 10 volumes of lysis buffer and centrifuging at 22,000×*g* for 15 min at 4 °C to obtain membranes and 10 % lysate (cytosol) fraction. UnHEs were also lysed with a 1:2 ratio of lysis buffer and centrifuged at 35,000×*g* for 30 min to obtain a concentrated lysate (Fig. 1).

Preparation of Triton Shells from Erythrocyte Membranes and Their Treatment with Cytosol

UnHEMs or HEMs ($n = 4$) were mixed with an equal volume of Triton X-100 solution (4 % v/v Triton X-100, 10 mM

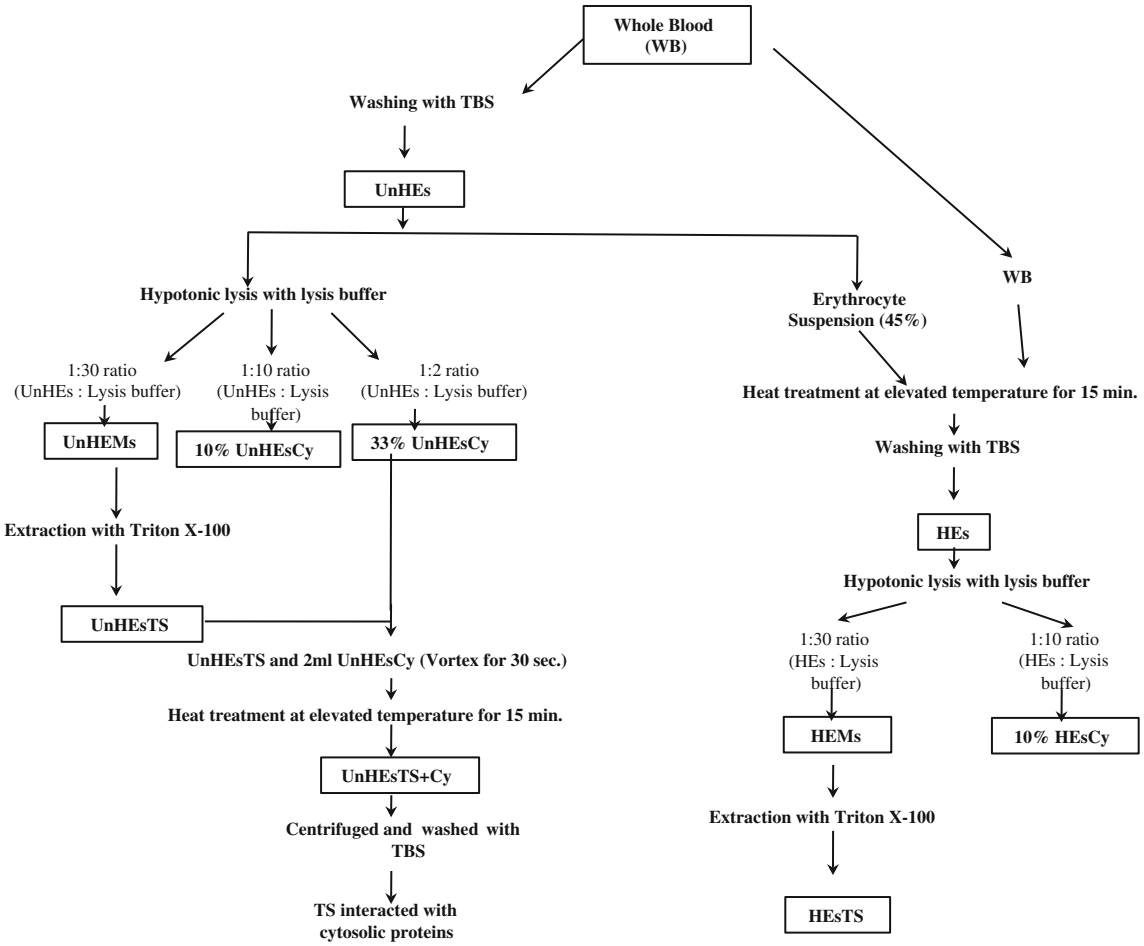

Fig. 1 Flowchart for sample preparation

Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM PMSF, 1 mM EDTA and 0.5 mM 2-mercaptoethanol) essentially as described by Gudi and Gupta (1993) with some modifications. After incubation for 20 min on ice, the extract was centrifuged at 10,000×*g* for 10 min in a refrigerated centrifuge. The pellet containing the Triton shells (TS) was separated and used for further studies. The Triton shells from unheated erythrocytes (UnHEsTS) were used to study the interactions between the membrane skeletal proteins in the Triton shells and the cytosolic proteins obtained from unheated erythrocytes (UnHEsCy). For this purpose, UnHEsTS were mixed with 2 ml of UnHEsCy and incubated at 44, 46, 48 and 50 °C in a water bath for 15 min. Suspensions of UnHEsTS and UnHEsCy were then cooled to RT and centrifuged at 22,000×*g* for 15 min at 4 °C. Pellets were washed twice with lysis buffer to remove extra cytosolic proteins.

Analysis of the Protein Profile of Erythrocytes, Their Lysates, Membranes and the Triton Shells

Protein in the different erythrocyte preparations was estimated by the procedure of Lowry et al. (1951). The protein profiles of the different preparations were analyzed by one-dimensional (1D) polyacrylamide gel electrophoresis (PAGE) in the presence of SDS essentially according to Laemmli (1970). Protein samples were solubilized in sample buffer containing 0.031 M Tris, 1 % SDS, 0.25 % β-mercaptoethanol and 5 % glycerol. After electrophoresis, the gels were fixed for 1 h in 40 % methanol–10 % acetic acid and then stained overnight in the same solution containing 0.1 % (w/v) Coomassie brilliant blue (CBB). Gels were destained in 40 % methanol–10 % acetic acid. The CBB-stained gel images were scanned using the Scanner HP scan jet 7400c (Hewlett Packard, Palo Alto, CA).

Gel Image Analysis

Densitometric analysis of CBB-stained bands on 1D gels was done using the UVP BioImaging Systems LabWorks™ Image Acquisition and Analysis software, version 4.0.0.8. The intensity of selected bands was measured and normalized by dividing with the total intensity of all the bands in the respective lanes.

Statistical Analysis

The relative intensity of each band from different samples was added and divided by the number of samples to obtain the relative mean intensity. The data from ten samples were then expressed as mean ± standard error (SE). Difference in the intensity of the bands between unheated and heated erythrocyte samples was determined using GraphPad (San Diego, CA) PRISM 5, version 5.03, for an analysis of variance followed by the unpaired, two-tailed t test. The significance level was set at $p < 0.05$.

Identification of Proteins by Mass Spectrometry

Protein bands of interest were cut out from CBB-stained gels of two different samples and processed for identification by mass spectrometry (MS) [matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF-TOF)] according to Shevchenko et al. (1996). The protein digest was premixed with an equal volume of CHCA matrix and spotted on a MALDI plate (Bruker Daltonik, Bremen, Germany). Peptide mass fingerprint (PMF) data was acquired on MALDI-TOF (Ultraflex II, Brucker Daltonik) in the reflector mode. Mass calibration was carried out using a peptide mixture spanning mass range of 800–4,000 *m/z* and error was kept to less than 10 ppm. The resulting PMF data were processed and further analyzed using BioTools Version 3.0 (Brucker Daltonik) software. The data were searched against Swiss-Prot database with *Homo sapiens* species using the MASCOT search engine. Only those proteins identified by MASCOT search criteria with the top significant score were considered as acceptable for further validation. The proteins identified were examined for sequence coverage, number of peptides matched, agreement between theoretical and experimental gel molecular weight and matching of major peaks of PMF with the peptides identified in the protein. The identity of each protein was further confirmed by MS–MS experiments.

Results

Heat Stress and Osmotic Fragility

Erythrocytes subjected to heat stress show the effect of different temperatures on osmotic fragility (Fig. 2). The osmotic fragility curves of HEs shifted to the right of the curve for UnHEs with a rise in temperature from 44 °C to 50 °C. This indicates cell instability with a rise in temperature. It has been reported earlier that denaturation of spectrin occurs at 49–50 °C (Yoshino and Minari 1987). To determine if the osmotic fragility is only due to this protein or whether other membranes/cytosolic proteins are involved, the cytosol and membranes from UnHEs and HEs were analyzed by SDS-PAGE, followed by identification of the proteins by MS.

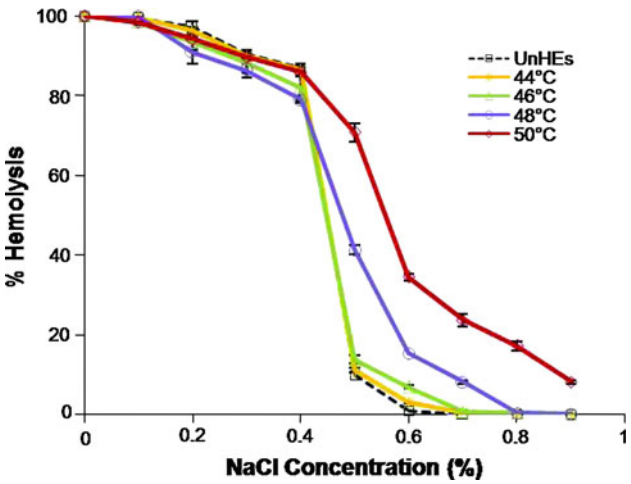

Fig. 2 Osmotic fragility curve obtained from unheated erythrocytes (*UnHEs*) and heated erythrocytes at various temperatures for 15 min. The data are mean ± SE of independent experiments ($n = 4$)

Analysis of the Protein Profile of Heat-Stressed Erythrocyte Membranes

A significant osmotic fragility (8.1 %, $p < 0.001$) at isotonic concentration (0.9 % NaCl) was observed only in erythrocytes heated at 50 °C for 15 min; thus, protein profiling of the erythrocyte membranes so treated was performed, and the bands obtained were subjected to densitometric analysis. Figure 3a shows the representative 1D protein profile, and Fig. 3b, c shows the densitometric analysis of the proteins from UnHEMs and HEMs obtained from erythrocytes of healthy individuals. The protein peak number in the densitogram corresponds to the band number shown in the 1D picture. The peak intensities of proteins band numbers 8, 9, 12 and 15–20 showed increased intensity after treatment. The major intensity changes were clearly observed with band numbers 15–18 ($p < 0.001$). All other proteins (band numbers 2–7, 11 and 13 with $p < 0.001$; 1 and 14 with $p < 0.01$; and 10 with p nonsignificant) showed a decrease in their intensities in HEMs.

Mass Spectrometry

The identity of the membrane proteins from UnHEs and HEs (50 °C for 15 min) was obtained by MS. The bands were cut from the 1D gel, and after tryptic digestion, the peptides were subjected to MALDI-TOF MS analysis. Proteins identified from UnHEMs and HEMs are given in Fig. 3a and Table 1. The major (bands 15–18) and some protein bands with minor changes in intensity (bands 8, 9, 19 and 20) were seen in HEMs (lane 2). These major and

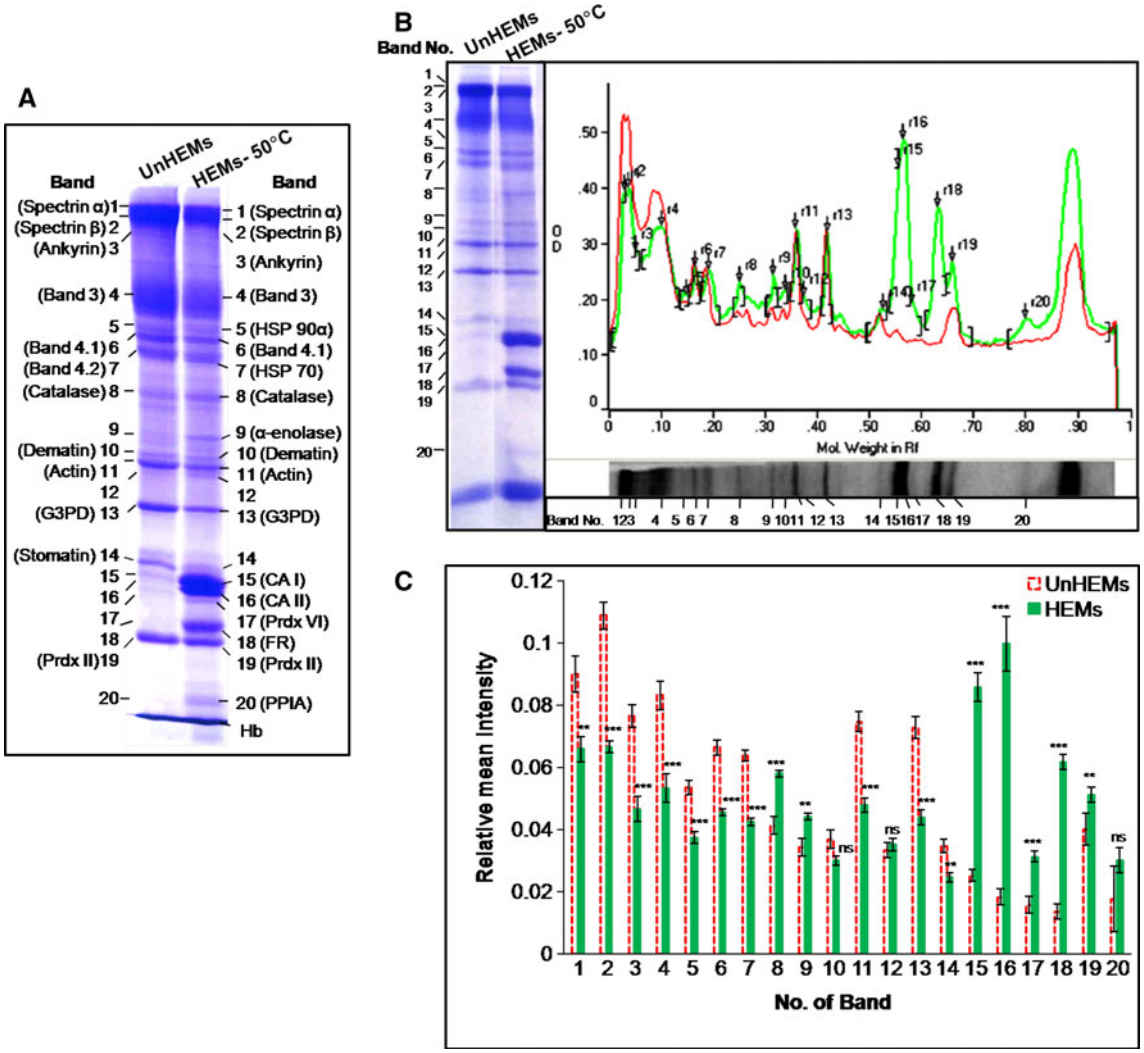

Fig. 3 Analysis of membrane proteins obtained from unheated (*UnHEMs*) and heat-stressed (*HEMs*) erythrocyte membranes. **a** Separation of membrane proteins obtained from UnHEMs and HEMs by SDS-PAGE (12 % gel) and stained with CBB followed by mass spectrometric identification (Table 1). *Lane 1* UnHEMs (60 μg); *lane 2* HEMs at 50 °C for 15 min (60 μg). **b** Densitometric spectra of UnHEMs (*red peaks*) and HEMs (*green peaks*) ($n = 1$). **c** Bar diagram showing changes in the intensity of the proteins ($n = 10$). The data are mean ± SE ($n = 10$). *Upper symbol* indicates the differences versus control (UnHEMs). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *ns* nonsignificant

minor altered/additional proteins were identified as erythrocyte cytosolic proteins: CA I, CA II, Prdx VI, FR, catalase, α-enolase, Prdx II and PPIA, respectively (Fig. 3a, lane 2, and Table 1). Band 12 which showed a slight increase in intensity was not identified by MS (Fig. 3a, lane 2). The two proteins catalase and Prdx II, which were present in HEMs, were also present in detectable amounts in the membranes obtained from UnHEs. Bands 5 and 7 showed less intensity ($p < 0.001$) in HEMs than UnHEMs (Fig. 3a), and these were identified as HSP90α and HSP70, respectively. Band 7 was identified adjacent to band 4.2 in HEMs, which was identified as protein band 4.2 in UnHEMs.

Exposure of Erythrocyte Suspension to Thermal Stress

The rapid increase in osmotic fragility of erythrocytes was observed with 0.5 % NaCl concentration at different elevated temperatures: 44, 46, 48 and 50 °C for 15 min (Fig. 2). Therefore, protein profiling of isolated and washed erythrocytes suspended in isotonic buffer (45 % cell suspension) was done after exposure to those elevated temperatures. Figure 4 shows the pattern of the whole erythrocyte (lane 1), lysate (cytosol, lanes 2–6) and membrane (lanes 7–11) proteins obtained from erythrocyte suspensions following heat stress at different elevated temperatures. The results were reproduced with blood

Table 1 Details of the mass spectrometric analysis for proteins mentioned in Fig. 2a

Band no.	Protein ID	Acc. no.	Theoretical M_r (kDa)/pI	Observed M_r (kDa)/pI	MS analysis			MS–MS analysis	
					Score[1]	Sequence coverage (%)	Peptides matched	Peptides	Score[1]
UnHEM									
1	Spectrin α	P02549	279.99/4.95	281/4.95	162	19	29/53	R.QYEQCLDFHLFYR.D	250
								K.VADDLLFEGLLTPEGAQIR.Q	
								K.ALGVPSSPYTWLTVEVLER.T	
								K.YSTIGLAQQWDQLYQLGLR.M	
2	Spectrin β	P11277	246.46/5.15	247/5.15	81	7	10/25	K.GNLEVLLFTIQSR.M	38
								K.FANSLTGVQQQLQAFSTYR.T	
3	Ankyrin	P16157	206.26/5.65	207/5.65	82	8	11/32	R.FWLSDCPR.T	286
								R.IITTDFPLYFVIMSR.L	
								K.TGFTPLHIAAHYENLNVAQLLLNR.G	
								R.IIALGPTGAQFLSPVIVEIPHFASHGR.G	
4	Band3	P02730	101.79/5.08	102/5.08	108	22	13/42	R.VLLPLIFR.N	318
								R.GWVIHPLGLR.S	
								R.ADFLEQPVLGFVR.L	
								R.LQEAAELEAVELPVPIR.F	
								R.FLFVLLGPEAPHIDYTQLGR.A	
6	Band 4.1	P11171	97.01/5.42	97.5/5.42	59	19	14/62	R.INRFPWPK.V	168
								K.IRPGEQEQYESTIGFK.L	
								K.NFMESVPEPRPSEWDKR.L	
								K.IRPGEQEQYESTIGFKLPSYR.A	
7	Band 4.2	P16452	77/8.39	77.8/8.39	102	26	14/47	K.WWSAVVEER.D	244
								R.GQPFTIILYFR.A	
								K.FQFTPTHVGLQR.L	
								K.IITIGLFFSNFER.N	
8	Catalase	P04040	59.75/6.9	59.9/6.9	68	30	11/67	R.AFYVNVLNEEQR.K	127
								R.LGPNYLHIPVNCPYR.A	
								R.GPLLVQDVVFTDEMAHFDRER.I	
10	Band 4.9 (dematin)	Q08495	45.51/8.94	45.6/8.94	74	24	8/27	R.SPGIISQASAPR.T	124
								K.STSPPPSPEVWADSR.S	
								R.TSLPHFHHPETSRPDSNIYK.K	
								K.IETDYWPCPPSLAVVETEWR.K	
								K.AILDIERPDLMIYEPHFTYSLLEHVELPR.S	

Table 1 continued

Band no.	Protein ID	Acc. no.	Theoretical M_r (kDa)/pI	Observed M_r (kDa)/pI	MS analysis			MS–MS analysis	
					Score[1]	Sequence coverage (%)	Peptides matched	Peptides	Score[1]
11	Actin	P60709	41.73/5.29	42/5.29	108	28	6/44	R.AVFPSIVGRPR.H	410
								K.IWHHTFYNELR.V	
								K.SYELPDGQVITIGNER.F	
								K.DLYANTVLSGGTTMYPGIADR.M	
								R.TTGIVMDSGDGVTHTVPIYEGYALPHAILR.L	
13	Glyceraldehyde-3-phosphate dehydrogenase (G3PD)	P04406	36.05/8.57	36.2/8.57	88	38	11/44	K.LVINGNPITIFQER.D	407
								K.LISWYDNEFGYSNR.V	
								K.VIHDNFGIVEGLMTTVHAITATQK.T	
								R.VIISAPSADAPMFVMGVNHEKYDNSLK.I	
14	Band 7 (stomatin)	P27105	31.73/7.71	31.8/7.71	64	30	8/44	K.GPGLFFILPCTDSFIK.V	81
								R.ALKEASMVITESPAALQLR.Y	
								R.ALKEASMVITESPAALQLR.Y	
								K.GPGLFFILPCTDSFIKVDMR.T	
								K.GPGLFFILPCTDSFIKVDMR.T	
19	Peroxiredoxin II (Prdx II)	P32119	21.89/5.66	22/5.66	115	41	8/51	R.GLFIIDGKGVLR.Q	491
								K.EGGLGPLNIPLLADVTR.R	
								R.KEGGLGPLNIPLLADVTR.R	
								K.EGGLGPLNIPLLADVTRR.L	
								K.LGCEVLGVSVDSQFTHLAWINTPR.K	
HEM									
1	Spectrin α	P02549	279.99/4.95	281/4.95	59	9	12/26	R.QYEQCLDFHLFYR.D	217
								K.VADDLLFEGLLTPEGAQIR.Q	
								K.ALGVPSSPYTWLTVEVLER.T	
								K.YSTIGLAQQWDQLYQLGLR.M	
2	Spectrin β	P11277	246.46/5.15	247/5.15	135	19	25/52	R.DLLSWMESIIR.Q	103
								K.GNLEVLLFTIQSR.M	
								R.LWSYLQELLQSR.R	
								K.FANSLTGVQQQLQAFSTYR.T	
3	Ankyrin	P16157	206.26/5.65	207/5.65	66	9	11/18	R.IITTDFPLYFVIMSR.L	135
								K.TGFTPLHIAAHYENLNVAQLLLNR.G	
								R.IIALGPTGAQFLSPVIVEIPHFASHGR.G	

Table 1 continued

Band no.	Protein ID	Acc. no.	Theoretical M_r (kDa)/pI	Observed M_r (kDa)/pI	MS analysis			MS–MS analysis	
					Score[1]	Sequence coverage (%)	Peptides matched	Peptides	Score[1]
4	Band 3	P02730	101.79/5.08	102/5.08	67	20	14/45	R.GWVIHPLGLR.S	333
								R.LQEAAELEAVELPVPIR.F	
								K.HSHAGELEALGGVKPAVLTR.S	
								R.FIFEDQIRPQDREELLR.A	
								R.FLFVLLGPEAPHIDYTQLGR.A	
5	Heat shock protein 90α (HSP90α)	P07900	84.66/4.94	85/4.94	69	25	13/58	R.RAPFDLFENR.K	44
								K.HFSVEGQLEFR.A	
								K.LDSGKELHINLIPNK.Q	
6	Band 4.1	P11171	97.01/5.42	97.5/5.42	91	17	12/44	R.INRFPWPK.V	117
								R.QASALIDRPAPHFER.T	
								K.IRPGEQEQYESTIGFK.L	
								K.NFMESVPEPRPSEWDKR.L	
7	Heat shock protein 70 (HSP70)	P08107	70.05/5.48	77.8/8.39	56	23	9/31	K.QTQIFTTYSDNQPGVLIQVYEGER.A	23
8	Catalase	P04040	59.75/6.9	59.9/6.9	115	36	14/62	R.LFAYPDTHR.H	246
								R.AFYVNVLNEEQR.K	
								K.GAGAFGYFEVTHDITK.Y	
								R.LGPNYLHIPVNCPYR.A	
9	α-Enolase	P06733	47.16/7.01	47.5/7.0	57	23	8/31	K.LAMQEFMILPVGAANFR.E	118
								K.LAMQEFMILPVGAANFR.E	
								K.AGYTDKVVIGMDVAASEFFR.S	
10	Band 4.9 (dematin)	Q08495	45.51/8.94	45.6/8.94	62	19	5/21	K.STSPPPSPEVWADSR.S	80
								R.TSLPHFHHPETSRPDSNIYK.K	
								K.STSPPPSPEVWADSRSPGIISQASAPR.T	
								K.AILDIERPDLMIYEPHFTYSLLEHVELPR.S	
11	Actin	P60709	41.73/5.29	42/5.29	69	36	11/55	R.AVFPSIVGRPR.H	401
								K.IWHHTFYNELR.V	
								K.SYELPDGQVITIGNER.F	
								K.DLYANTVLSGGTTMYPGIADR.M	
								R.TTGIVMDSGDGVTHTVPIYEGYALPHAILR.L	
13	Glyceraldehyde-3-phosphate dehydrogenase(G3PD)	P04406	36.05/8.57	36.2/8.57	68	25	6/20	K.LVINGNPITIFQER.D	175
								K.LISWYDNEFGYSNR.V	
								K.VIHDNFGIVEGLMTTVHAITATQK.T	
								R.VIISAPSADAPMFVMGVNHEKYDNSLK.I	

Table 1 continued

Band no.	Protein ID	Acc. no.	Theoretical M_r (kDa)/pI	Observed M_r (kDa)/pI	MS analysis			MS–MS analysis	
					Score[1]	Sequence coverage (%)	Peptides matched	Peptides	Score[1]
15	Carbonic anhydrase I (CA I)	P00915	28.87/6.59	28.9/6.59	101	37	9/20	K.GGPFSDSYR.L	300
								K.EIINVGHSFHVNFEDNDNR.S	
								K.EIINVGHSFHVNFEDNDNRSVLK.G	
								R.SLLSNVEGDNAVPMQHNNRPTQPLK.G	
								R.SLLSNVEGDNAVPMQHNNRPTQPLKGR.T	
16	Carbonic anhydrase II (CA II)	P00918	29.24/6.87	29.2/6.87	150	60	15/50	K.SADFTNFDPR.G	198
								K.YAAELHLVHWNTK.Y	
								K.YDPSLKPLSVSYDQATSLR.I	
								R.KLNFNGEGEPEELMVDNWRPAQPLK.N	
17	Peroxiredoxin VI (Prdx VI)	P30041	25.03/6.0	25.1/6.0	58	34	5/23	R.FHDFLGDSWGILFSHPR.D M.PGGLLLGDVAPNFEANTTVGR.I	212
								R.IRFHDFLGDSWGILFSHPR.D	
18	Flavin reductase (FR)	P30043	22.11/7.13	22.2/7.13	231	86	19/38	K.HDLGHFMLR.C	248
								K.TVAGQDAVIVLLGTR.N	
								K.VVACTSAFLLWDPTKVPPR.L	
								R.LPSEGPRPAHVVVGDVLQAADVDK.T	
								K.YVAVMPPHIGDQPLTGAYTVTLDGR.G	
19	Peroxiredoxin II (Prdx II)	P32119	21.89/5.66	22/5.66	126	41	8/32	R.GLFIIDGKGVLR.Q	449
								K.EGGLGPLNIPLLADVTR.R	
								R.KEGGLGPLNIPLLADVTR.R	
								K.EGGLGPLNIPLLADVTRR.L	
								K.LGCEVLGVSVDSQFTHLAWINTPR.K	
20	Peptidyl-prolyl *cis–trans* isomerase A (PPIA)	P62937	18.01/7.68	18.2/7.68	57	42	6/29	R.IIPGFMCQGGDFTR.H	104
								R.IIPGFMCQGGDFTR.H	
								K.SIYGEKFEDENFILK.H	
								M.VNPTVFFDIAVDGEPLGR.V	

Acc. no. accession number

[1] MS and MS–MS scores are mentioned in rounded figures

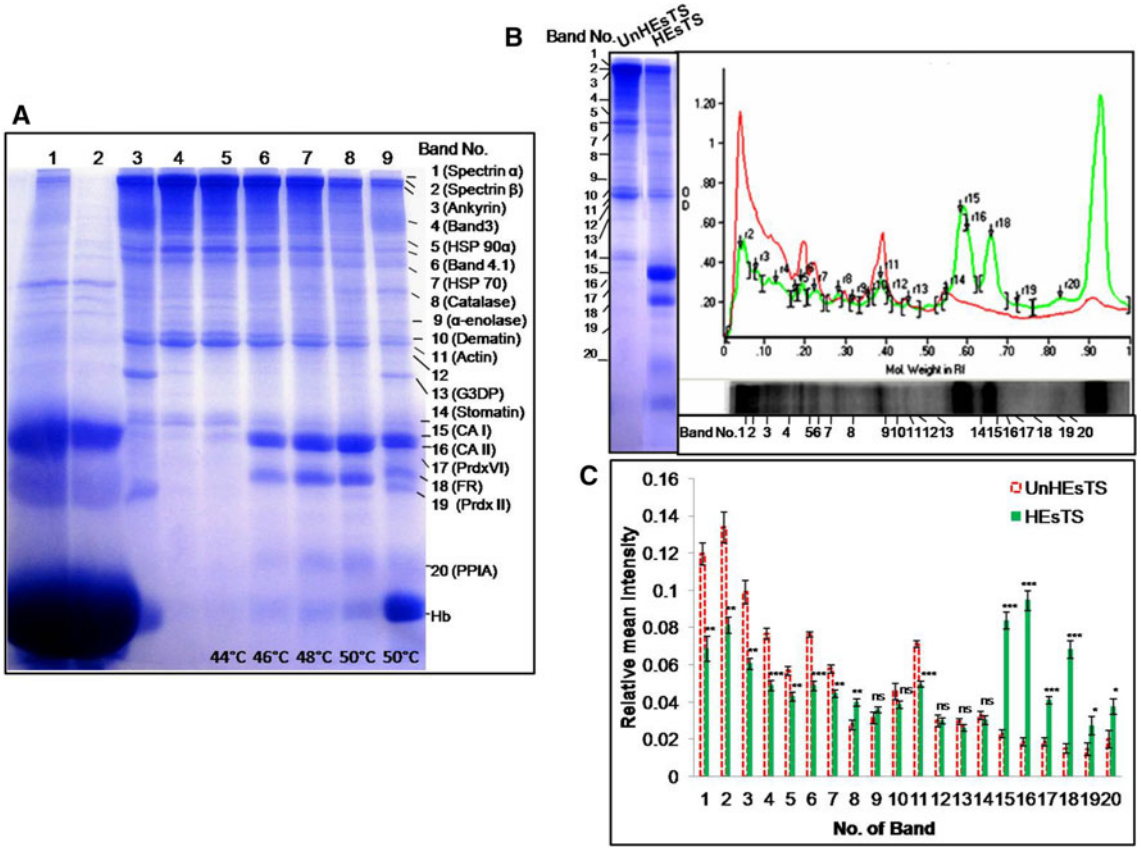

Fig. 4 Analysis of erythrocyte lysate (cytosol) and membrane proteins obtained from erythrocyte cell suspension exposed to various temperatures by SDS-PAGE (12 % gel) and stained with CBB. *Lane 1* unheated erythrocytes (whole cells, *UnHEs*); *lane 2* unheated erythrocyte lysate (*UnHEsCy*); *lanes 3–6* lysates (*HEsCy*) obtained from heat-stressed erythrocytes exposed to 44, 46, 48 and 50 °C for 15 min, respectively (60 µl protein in each lane); *lane 7* unheated erythrocyte membrane (*UnHEMs*); *lanes 8–11* erythrocyte membranes (*HEMs*) obtained from heat-stressed erythrocytes exposed to 44, 46, 48 and 50 °C for 15 min, respectively (60 µg protein in each *lane*). This is a representative pattern obtained for four different samples

samples from four different donors. The appearance of altered/additional bands with increased intensities was associated with the membranes prepared from erythrocytes heated at 46–50 °C (lanes 9–11). These additional bands were identified as cytosolic proteins which become associated with membrane proteins at and above 46 °C, corresponding to the osmotic fragility curves of erythrocytes (Fig. 2). The translocation of CA I and II (bands 15 and 16) was also observed at a much lower amount at 44 °C, and the first clear intense appearance of CA I, CA II and FR (bands 15, 16 and 18) was observed at 46 °C (lane 9). The intensities of these bands increased with further rises in temperature to 48 and 50 °C (lanes 10 and 11). The presence of minor changes in intensity of other proteins—catalase, α-enolase, Prdx VI, Prdx II and PPIA—was also observed with a rise in temperature (lanes 10 and 11) in HEMs and was absent in UnHEMs (lane 7) at their corresponding positions except for catalase and Prdx II. All these identified proteins in HEMs had corresponding bands in the UnHEsCy (lane 2). The localization of cytosolic and membrane proteins in the erythrocyte correlated well with the erythrocyte proteome analysis (Kakhniashvili et al. 2004; Pasini et al. 2006; Goodman et al. 2007), where cytosolic and membrane proteins are listed separately. A decrease in the intensity of proteins—spectrin α, spectrin β, ankyrin, band 3, HSP90α, band 4.1, HSP70, dematin, actin, glyceraldehyde-3-phosphate dehydrogenase (G3PD) and stomatin (Fig. 4, lanes 9–11)—was also similar to that seen in HEMs (Fig. 3b, c). Also, Hb is associated with HEMs, and this association increases with a rise in temperature (Fig. 4, lanes 8–11).

Exposure of Whole Blood to Thermal Stress

As exposure of washed erythrocyte suspensions showed significant changes in the protein pattern of erythrocyte membranes, whole blood was also exposed to different elevated temperatures—44, 46, 48 and 50 °C—for 15 min to determine whether the same effect of temperature is seen with erythrocytes when they are present in whole blood. The protein profiling of the whole erythrocytes (unheated), lysate (cytosol) and membrane proteins following heat

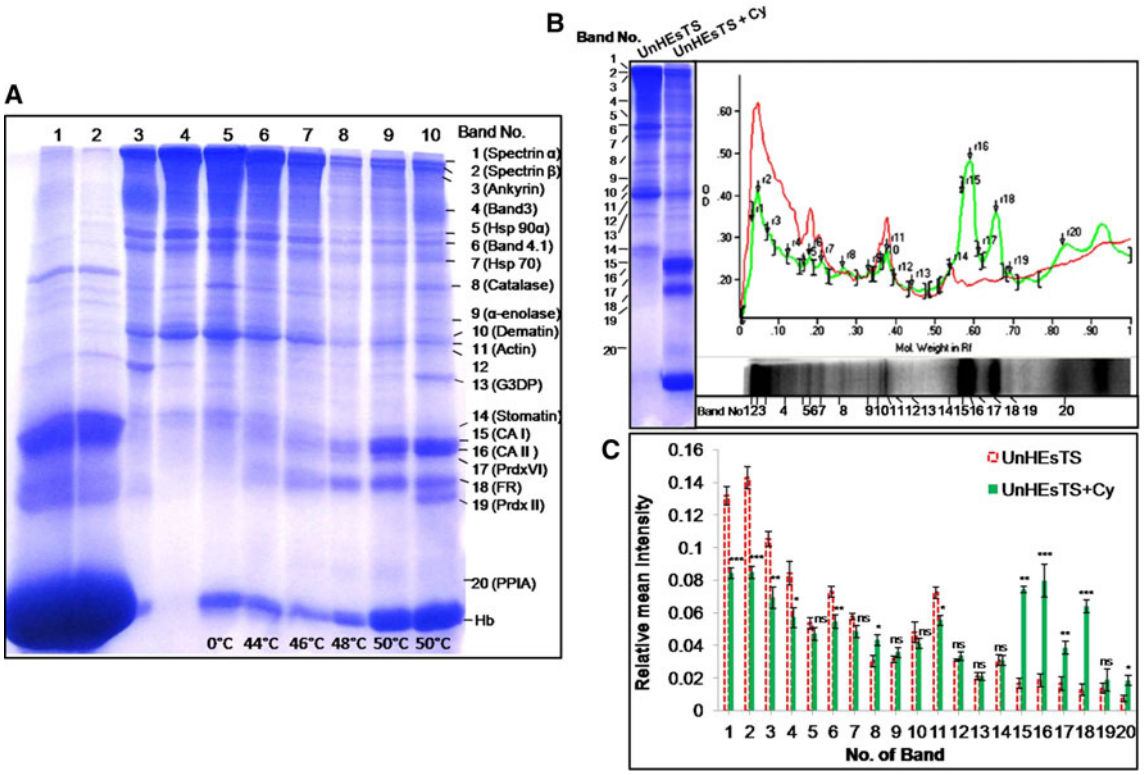

Fig. 5 Analysis of Triton shells obtained from erythrocyte membranes exposed to various temperatures. **a** Separation of proteins of Triton shells obtained from unheated (*UnHEMs*) and heat-stressed (*HEMs*) erythrocyte membranes by SDS-PAGE (12 % gel) and stained with CBB followed by mass spectrometric identification (Table 2). *Lane 1* unheated erythrocytes (whole cells, *UnHEs*, 60 µl); *lane 2* unheated erythrocyte lysate (*UnHEsCy*, 60 µl); *lane 3* unheated erythrocyte membrane (*UnHEMs*, 60 µg); *lanes 4–8* Triton shells obtained from unheated (*UnHEsTS*) and heat-stressed (*HEsTS*) erythrocytes exposed to 44, 46, 48 and 50 °C for 15 min, respectively (50 µg protein in each *lane*); *lane 9* membranes (*HEMs*) from heat-stressed erythrocytes exposed to 50 °C for 15 min (60 µg). **b** Densitometric spectra of UnHEsTS (*red peaks*) and HEsTS (*green peaks*). **c** *Bar* diagram shows changes in protein intensity ($n = 4$). The data are mean ± SE ($n = 4$). *Upper symbol* indicates the differences versus control (UnHEsTS). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *ns* nonsignificant (Color figure online)

stress at different elevated temperatures was done (data not shown); and similar results to those given above (Fig. 4) were observed. The results were reproducible with blood samples from four different donors.

Analysis of the Protein Profile of Triton Shells

To identify the location of the translocated proteins in the membrane (i.e., peripheral or integral), UnHEMs and HEMs were extracted with 4 % Triton X-100. When erythrocyte membranes are extracted with Triton X-100, most of the lipids and other proteins such as band 3, band 4.5 and band 6 are extracted, leaving behind a structure known as the "cytoskeleton." Figure 5a shows the protein pattern of the whole erythrocytes (lane1), UnHEsCy (lysate/cytosol, lane 2), UnHEMs (lane 3), HEMs, 50 °C (lane 9) and UnHEsTS (lane 4) and those (HEsTS) exposed to heat stress at 44, 46, 48 and 50 °C for 15 min (lanes 5–8, respectively). The selected proteins identified from heated erythrocyte Triton shells (HEsTS) are given in Table 2. The Triton shell consists of spectrin (α and β), ankyrin, 4.1, 4.2, dematin, actin and stomatin as well as 15–25 % of band 3 (band numbers 1, 2, 3, 6, 7, 10, 11, 14 and 4 respectively). Figure 5b and the bar diagram (Fig. 5c) show the densitometric comparison of UnHEsTS and HEsTS at 50 °C for 15 min. The bar diagram in Fig. 5c shows the relative mean intensity of proteins of UnHEsTS and HEsTS ($n = 4$). The intensity of protein bands 8, 9 and 15–20 in HEsTS was increased compared to HEMs (Fig. 4, lanes 9–11). The major changes in intensity were observed in lower–molecular weight membrane skeletal proteins, bands 15–18 ($p < 0.001$). Bands 8, 9, 19 and 20 were present in very low amounts in UnHEsTS compared to HEsTS (Fig. 5b, lanes 1 and 2, respectively). These protein bands were increased (insignificantly), and only band 20 showed a significant increase ($p < 0.05$) as seen in HEMs (Fig. 3a, lane 2). Bands 8 and 19 were present in UnHEMs (Fig. 4, lane 7) but were not observed in Triton shells extracted from UnHEMs (Fig. 5a, lane 4). At the same time, all other proteins (bands 4, 6 and 11 with $p < 0.001$;

Table 2 Details of the mass spectrometric analysis for proteins shown in Fig. 4b

Band no.	Protein ID	Acc. no.	Theoretical M_r (kDa)/pI	Observed M_r (kDa)/pI	MS analysis			MS–MS analysis	
					Score[1]	Sequence coverage (%)	Peptides matched	Peptides	Score[2]
HEsTS									
5	Heat shock protein 90α (HSP90α)	P07900	84.66/4.94	85/4.94	80	23	11/33	R.GVVDSEDLPLNISR.E	17
								R.VFIMDNCEELIPEYLNFIR.G	
7	Heat shock protein 70(HSP70)	P16452	70.05/5.48	77.8/8.39	70	20	8/26	K.QTQTFTTYSDNQPGVLIQVYEGER.A	27
8	Catalase	P04040	59.75/6.9	59.9/6.9	74	29	13/42	R.AFYVNVLNEEQR.K	142
								R.LGPNYLHIPVNCPYR.A	
								R.GPLLVQDVVFTDEMAHFDR.E	
								R.GPLLVQDVVFTDEMAHFDRER.I	
15	Carbonic anhydrase I (CA I)	P00915	28.87/6.59	28.9/6.59	126	37	8/23	K.GGPFSDSYR.L	217
								K.EIINVGHSFHVNFEDNDNR.S	
								K.EIINVGHSFHVNFEDNDNRSVLK.G	
16	Carbonic anhydrase II (CA II)	P00918	29.24/6.87	29.2/6.87	105	40	9/32	K.SADFTNFDPR.G	313
								K.YAAELHLVHWNTK.Y	
								K.YDPSLKPLSVSYDQATSLR.I	
								K.LNFNGEGEPEELMVDNWRPAQPLK.N	
								R.KLNFNGEGEPEELMVDNWRPAQPLK.N	
17	Peroxiredoxin VI (Prdx VI)	P30041	25.03/6.0	25.1/6.0	56	34	5/25	R.FHDFLGDSWGILFSHPR.D	144
								M.PGGLLLGDVAPNFEANTTVGR.I	
								R.IRFHDFLGDSWGILFSHPR.D	
18	Flavin reductase (FR)	P30043	22.11/7.13	22.2/7.13	154	71	13/43	K.TVAGQDAVIVLLGTR.N	282
								R.VISKHDLGHFMLR.C	
								K.VVACTSAFLLWDPTKVPPR.L	
								K.YVAVMPPHIGDQPLTGAYTVTLDGR.G	
								K.YVAVMPPHIGDQPLTGAYTVTLDGRGPSR.V	
19	Peroxiredoxin II (Prdx II)	P32119	21.89/5.66	22/5.66	118	48	10/51	K.EGGLGPLNIPLLADVTR.R	577
								R.KEGGLGPLNIPLLADVTR.R	
								K.EGGLGPLNIPLLADVTRR.L	
								R.LSEDYGVLKTDEGIAYR.G	
								K.LGCEVLGVSVDSQFTHLAWINTPR.K	

Table 2 continued

Band no.	Protein ID	Acc. no.	Theoretical M_r (kDa)/pI	Observed M_r (kDa)/pI	MS analysis			MS–MS analysis	
					Score[1]	Sequence coverage (%)	Peptides matched	Peptides	Score[2]
20	Peptidyl-prolyl *cis–trans* isomerase A(PPIA)	P62937	18.01/7.68	18.2/7.68	89	74	9/57	K.VKEGMNIVEAMER.F R.IIPGFMCQGGDFTR.H R.IIPGFMCQGGDFTR.H K.SIYGEKFEDENFILK.H M.VNPTVFFDIAVDGEPLGR.V	289

Acc. no. accession number

[1] MS and MS–MS scores are mentioned in rounded figures

1–3, 5 and 7 with $p < 0.01$; 10, 13 and 14 with p nonsignificant) showed a decrease in intensity.

Association of Cytosolic Proteins with Triton Shells

The experiments described above showed that most of the protein bands (HSP90α, HSP70, catalase, α-enolase, CA I, CA II, Prdx VI, FR and PPIA) which are associated with HEMs were present in the Triton shells—"membrane skeleton" (Fig. 5a, lanes 5–8, and their identities are given in Table 2). The decrease in intensity of stomatin continued (Fig. 5a, lanes 5–7). Proteins, catalase and Prdx II were not associated with UnHEsTS (Fig. 5a, lane 4). The Triton shells extracted from HEMs showed some catalase content at 48 and 50 °C (Fig. 5a, lanes 7 and 8), and Prdx II was observed only in a lesser amount in HEsTS at 50 °C (Fig. 5a, lane 8). Hemoglobin did not associate with HEsTS (Fig. 5a, lanes 5–8), as seen for HEMs (Fig. 4, lane 8–11).

Analysis of the Association of Cytosolic Proteins with Triton Shells

To determine how the different cytosolic proteins interact with the skeletal proteins, UnHEsTS were treated with UnHEsCy, and the combination (UnHEsTS + UnHEsCy) was mixed well and kept at 0, 44, 46, 48 and 50 °C for 15 min. Figure 6 shows the protein profile of unheated erythrocytes (whole cells, lane 1), lysate (lane 2), membrane (lane 3), Triton shells from unheated membranes (lane 4), Triton shells (derived from unheated erythrocyte membranes) incubated with the unheated lysate at 0, 44, 46, 48 and 50 °C for 15 min, respectively (lanes 5–9) and membranes from heat-stressed erythrocytes exposed to 50 °C for 15 min (lane 10). Figure 6b shows a densitometric pattern of a representative protein band profile of UnHEsTS and UnHEsTs + Cy. Figure 6c shows a bar diagram of the relative mean intensity of the bands from four samples.

The pattern is similar to that obtained for HEsTS (Fig. 5). Bands 8, 9, 12 and 15–20 in UnHEsTS + Cy were increased as in case of HEsTS (Fig. 5). Bands 15 and 17 ($p < 0.01$) and 16 and 18 ($p < 0.001$) showed maximum increases in intensity. A slight increase in intensity was observed for bands 9, 12 and 19; but the increase in intensity of bands 8 and 20 was significant ($p < 0.05$).

Interaction of Cytosolic Proteins and Triton Shells

The Triton shells from UnHEMs show the membrane skeletal protein bands seen in Fig. 5a, lane 4. The proteins which associate with the TS when UnHEsTS + UnHEsCy are heated to different temperatures (Fig. 6a, lanes 6–9) show similar results as obtained when Triton shells are made from HEMs (Fig. 5a, lanes 5–8), indicating that the interaction of

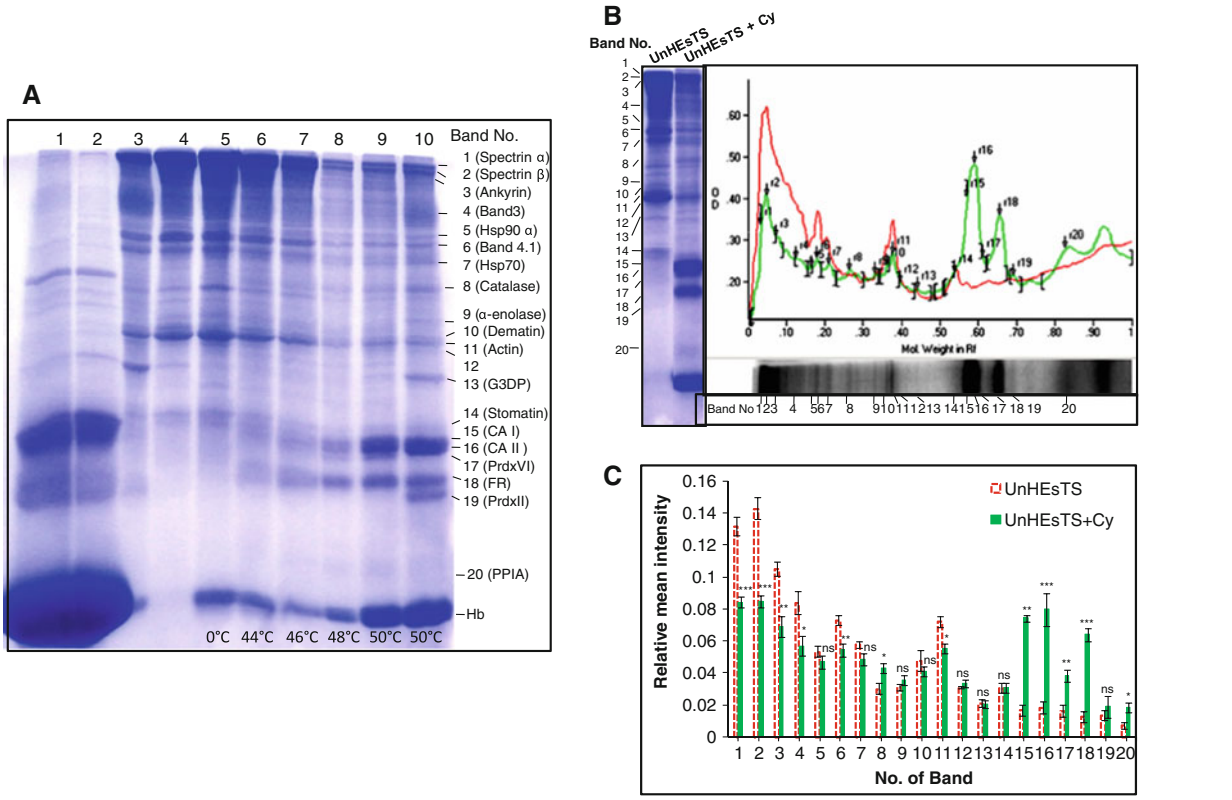

Fig. 6 Analysis of interactions of Triton shells and cytosolic proteins obtained from unheated erythrocytes (*UnHEs*) under heat stress. **a** Separation of proteins of Triton shells interacted with cytosolic proteins under heat stress by SDS-PAGE (12 % gel) and stained with CBB followed by mass spectrometric identification (Table 3). *Lane 1* unheated erythrocytes (whole cells, 60 µl); *lane 2* unheated erythrocyte lysate (60 µl); *lane 3* unheated erythrocyte membrane (60 µg); *lane 4* Triton shells obtained from unheated erythrocyte membrane (50 µg); *lanes 5–9* Triton shells (derived from unheated erythrocyte membranes) were incubated with the unheated lysate at 0, 44, 46, 48 and 50 °C for 15 min, respectively (50 µg protein in each *lane*); *Lane 10* Membranes from heat-stressed erythrocytes exposed to 50 °C for 15 min. (60 µg). **b** Densitometric spectra of UnHEsTS (*red peaks*) and UnHEsTS + Cy (*green peaks*). **c** *Bar* diagram shows changes in protein intensity ($n = 4$). The data are mean ± SE ($n = 4$). *Upper symbol* indicates the differences versus control (UnHEsTS). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; *ns* nonsignificant (Color figure online)

cytosolic proteins with the membrane skeleton proteins is specific. It is noted that proteins CA I, CA II, Prdx VI and FR start associating with the UnHEsTS when the combination is heated at 46 °C (Fig. 6a, lane 7). Further, catalase and Prdx II did not become associated with UnHEsTS when UnHEsTs were mixed with UnHEsCy and heated (Fig. 6a, lanes 6–9) or Triton shells were made from HEMs (Fig. 5a, lanes 6–8). As these proteins did not become attached with the Triton shells, they probably interact with the membrane protein/lipid bilayer not present in the Triton shells. Hb remained associated with UnHEsTS + Cy as expected since UnHEsTS is treated with the cytosol which does not have any Triton in it (Fig. 6a, lanes 5–9).

Discussion

There are few reports regarding the association of cytosolic proteins with the membrane of some mammalian erythrocytes exposed to heat stress (Gudi and Gupta 1993; Wagner et al. 2004; Rocha et al. 2009). In rhesus erythrocytes, HSP70-like protein together with other cytosolic proteins is reported to become associated with the membrane during heat stress (Gudi and Gupta 1993). Wagner et al. (2004) have reported the translocation of TCP 1, CA and FR from the cytosol to the membrane of human erythrocytes after exposure to elevated temperature. Our observations indicate that CA (isoforms I and II) and FR along with Prdx VI and PPIA become significantly associated with membranes. A minor increase in catalase and α-enolase content was also persistent. In addition, a specific association of these cytosolic proteins except catalase and Prdx II to the Triton-generated membrane shell has been shown. The new interesting observation about the interactions of cytosolic and cytoskeletal proteins is that cytosolic proteins become associated with shell proteins when UnHEsTS is heated with UnHEsCy at different elevated temperatures. The initial temperature of the association of cytosolic proteins with the erythrocyte membrane is 46 °C, and it increases to 50 °C.

Table 3 Details of the mass spectrometric analysis for proteins shown in Fig. 5b

Band no.	Protein ID	Acc. no.	Theoretical M_r (kDa)/pI	Observed M_r (kDa)/pI	MS analysis			MS–MS analysis	
					Score[1]	Sequence coverage (%)	Peptides matched	Peptides	Score[1]
UnHEsTS + Cy									
5	Heat shock protein 90α (HSP90α)	P07900	84.66/4.94	85/4.94	57	17	8/26	[2]	
7	Heat shock protein 70 (HSP70)	P16452	70.05/5.48	77.8/8.39	73	27	10/25	[2]	
8	Catalase	P04040	59.75/6.9	59.9/6.9	36	14	5/28	R.LGPNYLHIPVNCPYR.A R.GPLLVQDVVFTDEMAHFDRER.I	66
9	α-Enolase	P06733	47.16/7.01	47.5/7.0	33	18	6/18	K.LAMQEFMILPVGAANFR.E	33
15	Carbonic anhydrase I (CA I)	P00915	28.87/6.59	28.9/6.59	114	37	7/12	K.GGPFSDSYR.L K.EIINVGHSFHVNFEDNDNR.S R.SLLSNVEGDNAVPMQHNNRPTQPLK.G	113
16	Carbonic anhydrase II (CA II)	P00918	29.24/6.87	29.2/6.87	72	37	9/34	R.LIQFHFHWGSLDGQGSEHTVDK.K K.LNFNGEGEPEELMVDNWRPAQPLK.N R.KLNFNGEGEPEELMVDNWRPAQPLK.N	102
17	Peroxiredoxin VI (Prdx VI)	P30041	25.03/6.0	25.1/6.0	52	35	6/27	R.FHDFLGDSWGILFSHPR.D M.PGGLLLGDVAPNFEANTTVGR.I R.IRFHDFLGDSWGILFSHPR.D	359
18	Flavin reductase (FR)	P30043	22.11/7.13	22.2/7.13	61	30	5/18	R.LQAVTDDHIR.M R.VISKHDLGHFMLR.C K.YVAVMPPHIGDQPLTGAYTVTLDGR.G	51
19	Peroxiredoxin II (Prdx II)	P32119	21.89/5.66	22/5.66	114	41	9/32	R.QITVNDLPVGR.S K.EGGLGPLNIPLLADVTR.R R.KEGGLGPLNIPLLADVTR.R K.EGGLGPLNIPLLADVTRR.L K.LGCEVLGVSVDSQFTHLAWINTPR.K	321
20	Peptidyl-prolyl *cis–trans* isomerase A (PPIA)	P62937	18.01/7.68	18.2/7.68	85	70	9/46	R.IIPGFMCQGGDFTR.H K.SIYGEKFEDENFILK.H M.VNPTVFFDIAVDGEPLGR.V	111

Acc. no. accession number

[1] MS and MS–MS scores are mentioned in rounded figures

[2] MS-MS data could not be obtained for HSP90α and HSP70

Upon exposure to elevated temperatures, a number of transitions occur in the erythrocyte membrane. Spectrin is the least thermostable major membrane protein and undergoes a major denaturation at 49–50 °C during the first thermal transition (Brandts et al. 1977). This leads to destabilization of the membrane skeleton and results in the formation of spherocytes (Coakley et al. 1979; Lysko et al. 1981; Eskelinen et al. 1985). Similar results were obtained when intact erythrocytes were exposed to elevated temperatures, and a direct correlation between hemolysis and membrane protein denaturation has been reported (Lepock et al. 1989). Heat-stressed erythrocytes vesiculate (Smith and Palek 1983) and show reduced deformability (Mohandas and Gallagher 2008). Parameters of the protein structure of spectrin studied as circular dichroism, scanning calorimetry (Brandts et al. 1977), fluorescence polarization (Yoshino and Marchesi 1984) and trypsin susceptibility (Knowles et al. 1983) indicated that clear structural transitions occurred at temperatures around 49 °C. Solubilized spectrin in membrane skeletal extract when exposed to heat treatment experiences much greater effects (Yoshino and Minari 1987) than in intact cells (Gudi et al. 1990). Although denaturation of spectrin is reported to occur at 49–50 °C, our observations indicate that translocation of cytosolic proteins to the membrane starts at 46 °C and increases progressively up to 50 °C. This indicates that some structural alterations in the spectrin molecules may be initiated at 46 °C, sufficient to cause interactions with cytosolic proteins. It is possible that the process of spectrin denaturation may start at 46 °C and only a small fraction gets denatured, while at 50 °C a larger fraction undergoes denaturation, which leads to increased fragility and recordable thermal transition effects. Studies on the effect of chemical modification of membrane proteins have indicated that both the alkylation and oxidation of thiol groups and subsequent heating make the membrane irreversibly more disorganized. Alkylation of thiol groups by *N*-ethylmaleimide (NEM) treatment decreases the heat denaturation temperature of spectrin and the fragmentation temperature of erythrocytes (Smith and Palek 1983). The oxidation of the erythrocyte membrane by diamide is related to the formation of disulfide bonds (Becker et al. 1986). Diamide as well as NEM treatment affect spectrin self-association in the membrane, leading to decreased association between dimers and, consequently, a looser skeletal organization (Streichman et al. 1988). The extent of thermal sensitivity depends on both the temperature and the state of the membrane thiol groups, and hyperthermia progressively decreases the levels of thiol groups in pig erythrocytes (Jozwiak et al. 1991). Membranes or Triton shells obtained from heat-stressed erythrocytes were analyzed by SDS-PAGE in the presence and absence of β-mercaptoethanol. The results indicate that no disulfide bond formation occurs between membrane and cytosolic proteins because additional protein complexes could not be observed in the heat-stressed samples without β-mercaptoethanol (results not shown). Extraction of heated erythrocytes or membranes isolated from HEs with non-ionic detergent Triton X-100 does not cause solubilization of cytosolic proteins. We have also observed that cytosolic proteins which are associated with Triton shells during heat treatment are not extracted with 0.5 M NaCl (results not given).

In conclusion, this study demonstrates the presence of cytosolic proteins in the membrane or membrane skeleton of heat-stressed erythrocytes. Such an association of cytosolic proteins with membranes or the membrane skeleton might be helpful in the stabilization of erythrocytes and in reducing the adverse effects on cells during thermal stress. It is likely that during summer in tropical countries the erythrocytes of individuals exposed to high temperatures for a longer time may become affected. A fraction of cytosolic proteins associated with membranes may serve as a marker of damage to body cells during thermal stress. Knowledge of the erythrocyte responses to heat stress helps in understanding the mechanism and provides avenues toward preventative measures for heat stress–related aftereffects.

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