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In Vivo Pharmaco-Proteomic Analysis of Hydroxyurea Induced Changes in the Sickle Red Blood Cell Membrane Proteome

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Abstract

Hydroxyurea (HU) is an effective drug for the treatment of sickle cell disease (SCD). The main clinical benefit of HU is thought to derive from its capacity to increase fetal hemoglobin (HbF) production. However, other effects leading to clinical benefit, such as improved blood rheology, have been suggested. In order to understand HU-induced changes at the proteomic level, we profiled sickle RBC membranes from of HU-treated and untreated patients. Our previous *in vitro* profiling studies on sickle RBC membranes identified a significant increase in predominantly anti-oxidant enzymes, protein repair and degradation components and a few RBC cytoskeletal proteins. In the present study, using 2D-DIGE (Two-Dimensional Difference In-Gel Electrophoresis) and tandem mass spectrometry, we detected 32 different proteins that significantly changed in abundance in the HU treatment group. The proteins that significantly increased in abundance were mostly membrane skeletal components involved in the regulation of RBC shape and flexibility, and those showing a significant decrease were components of the protein repair and degradation machinery. RBC palmitoylated membrane protein 55 (p55) is significantly increased in abundance at low (*in vitro*) and high (*in vivo*) concentrations of HU. Palmitoylated p55 may be an important target of HU-dependent regulation of the sickle RBC membrane, consistent with our earlier *in vitro* studies.

Keywords

Sickle Cell Disease; Hydroxyurea; Red Blood Cell Membrane; 2D- DIGE; Tandem Mass Spectrometry; Palmitoylated protein 55 (p55)

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1. Introduction

Sickle cell disease (SCD) is a recessive genetic disorder caused by a point mutation in the 6th codon of the β globin gene. At the amino acid level, this results in the substitution of glutamic acid by valine in the β-globin subunit of hemoglobin [1]. In the deoxygenated state, the mutant sickle hemoglobin (HbS) forms rigid and insoluble polymers that distort the shape of the RBCs giving them a characteristic sickle shape. The polymerization of HbS leads to the complex pathophysiology associated with SCD, which includes vaso-occlusion, chronic hemolysis and irreversible tissue damage [2].

HU is thought to be an effective drug for the management of SCD due to its capacity to increase HbF levels. Increased HbF levels inhibit the polymerization of HbS and reduce sickling [2]. However, the Multicenter Study of Hydroxyurea in Sickle Cell Anemia revealed that many patients showed clinical improvement before a significant rise in HbF levels [3]. Various reports have demonstrated that increase in HbF is not the only benefit of HU. Some of the factors involved in ameliorating the pathology of sickle cell disease after HU treatment are increased MCV of sickle cell RBCs [4], reduced adhesion of sickle cell RBCs to the endothelium [5] and increased deformability of sickle cell RBCs [6]. These findings point towards HU-induced alterations of additional cellular mechanisms that are yet to be identified and that may mediate the clinical benefits of HU. The understanding of these pathways and the drug mechanism warrants the need to identify additional sickle RBC membrane proteins whose expression is regulated by HU.

A previous *in vitro* protein profiling study performed in our laboratory identified significant increases in RBC anti-oxidant enzymes and protein repair and degradation components after exposure of sickle RBC membranes to low concentrations of HU (50 and 100 μM). Through this *in vitro* study, we further demonstrated that 50 μM HU exposed sickle RBC membranes showed a 2-fold increase in tyrosine phosphorylation of catalase as compared to counterparts not exposed to HU [7]. The *in vitro* protein profiling system allowed us to look at the same sickle RBC membrane sample from individual SS patients with and without HU exposure to identify dose-dependent proteomic changes *in vitro*, which is difficult to achieve in an *in vivo* clinical setting. However, the *in vitro* system utilizes mature enucleated RBCs that lack the capacity to synthesize new proteins and the *in vitro* proteomic changes identified mainly reflect post-translational modifications. Moreover, HU acts on late erythroid precursors in the bone marrow and influences the erythropoietic pathway [8]. Hence, in the present study, we have undertaken an *in vivo* proteomic analysis of sickle RBC membranes with the following aims: 1) Identify common HU-induced proteomic changes *in vitro* and *in vivo*, 2) Identify HU-induced changes at concentrations that are actually administered to SS patients in a clinical setting and 3) Identify changes in protein expression as well as protein modification.

Though some roles of HU in pathways other than HbF production have been reported, the protein targets altered in these pathways as a result of HU treatment are not known. With HU being the only FDA-approved drug to date, studies to investigate HU-dependent protein alterations are important to understand the drug's mechanism of action as well as its toxic and beneficial effects. With the goal of identifying RBC membrane protein alterations in homozygous sickle cell anemia (SS) patients on HU therapy, we performed 2D-DIGE followed by tandem mass spectrometry. A global protein profiling approach eliminates the need to study drug-induced response of individual cellular pathways and provides a common platform for the simultaneous fluorescent detection of thousands of drug-related changes in proteins. In this proteomic study, we report a significant increase in two major classes of proteins after *in vivo* HU therapy: RBC membrane skeletal components and glycolytic enzymes. A combination of 2D-DIGE and tandem mass spectrometry led to the identification of 32 different sickle RBC membrane proteins of interest showing a significant change in content as a response to an

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average dose of 35 mg/kg (400μM) administered *in vivo*. Thirty of these showed a significant increase and belonged to the group of RBC membrane skeletal proteins and glycolytic enzymes. The two proteins that showed a significant decrease represented components of the protein repair and degradation machinery. These results demonstrate some important HU-mediated proteomic changes, besides HbF production, likely to have implications in regulating sickle RBC rheology, metabolism and oxidative damage.

The significant increase in palmitoylated membrane protein 1 (p55) at low HU concentrations *in vitro* and high HU concentrations *in vivo* makes this protein an important response marker of HU therapy.

2. Materials and Methods

2.1. Subjects

After informed consent, human blood samples (10ml) were collected from homozygous SS patients by venipuncture using lithium heparin as an anti-coagulant at the Southwestern Comprehensive Sickle Cell Center. The treatment group included three adults and two children with SS who had received HU for at least 4 months. We selected these patients because they had an excellent clinical response to HU, based on a marked reduction in the frequency of hospitalizations for vaso-occlusive (painful) crises and acute chest syndrome as well as patient self-report (improved quality of life and decreased painful episodes at home). Untreated SS controls had not been treated at the time of phlebotomy or previously with HU. No patient from the treated or untreated groups had received a blood transfusion within the preceding 4 months.

2.2. Isolation of reticulocyte-free red blood cells

Reticulocyte free red blood cells were isolated by a density based method developed and assessed in our laboratory by Kakhniashvili et al. [9]. The collected blood samples were used within four hours. All the procedures were performed at room temperature. Five ml of blood was centrifuged at 550g for 10 minutes to remove plasma. The pelleted cells were washed four times with 10 volumes of PBS (11.9mM phosphate, pH 7.4, 137mM NaCl, 2.7mM KCl) by centrifuging at 550 g for 10 minutes. The washed blood cells were finally resuspended in PBS at 50% hematocrit and were loaded onto a single layer of 75% percoll of equal volume and centrifuged at 1000xg for 15 minutes in 15 ml centrifugal tubes in a bucket rotor. 75% percoll solution (GE Healthcare, p = 1.130±0.005g/ml) was prepared by dilution with 10X PBS and water. The bottom layer of the density gradient represents pure population of RBCs which after 3 washes of PBS, was used for membrane preparation.

2.3. Preparation of erythrocyte membranes

Erythrocyte membranes were prepared as described [10]. The RBCs were sedimented at 1000 *g* for 10 minutes at 4°C and resuspended in PBS (10mM $NaPO_4$, pH 7.6, 150mM NaCl). This step was repeated four times. The RBCs were then resuspended in 10 volumes of PBS and sedimented at 2000 *g* for 10 minutes. The washed RBCs were lysed in six volumes of lysis buffer (5mM $NaPO_4$, 1mM EDTA, pH 7.6) and were sedimented at 31,000 *g* for 30 minutes. This step was repeated until the pellet became white or light pink. Membrane protein concentration was measured by Protein assay reagent (Bio-Rad). The membranes containing ~ 4–6 mg of protein/ml were vacuum dried and solubilized in lysis buffer (30mM Tris-HCl, pH 8.5, 7M urea, 2M thiourea, and 2% (w/v) nonionic detergent ASB 14).

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2.4. Minimal labeling of SS membrane proteins

SS membrane proteins corresponding to 100 μg each of (−)HU and (+)HU samples solubilized in lysis buffer were minimally labeled with Cy3 and Cy5 fluorophores respectively according to the manufacturer's protocol (Amersham Biosciences).

2.5. Separation of proteins in first dimension (IEF)

100 μg each of the control and drug treated samples were mixed together and rehydration buffer (1% Pharmalyte 3–10 NL, 7 M urea, 2 M thiourea, 2 % ASB-14, 2 mg/ml DTT) was added to the final protein mixture. The sample, included in the rehydration solution was loaded on the IPG (Immobilized pH Gradient) strip holder. Immobiline DryStrip gel (Amersham Biosciences, pH gradient 3–10 Non-Linear, length of strip 13 cm) was placed over the sample and finally IPG cover fluid was added to minimize evaporation and urea crystallization. Rehydration of the IPG strip proceeded on an Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences) for 12 hours at 20°C. Isoelectric focusing was performed in three steps: at 500 V for 1 hour, at 1000 V for 1hour and at 8000 V for 33,000 Volt-hours.

2.6. Reduction and alkylation of the IEF separated proteins

Prior to running the second dimension, the Immobiline strip with separated proteins were equilibrated and reduced in a solution containing 50 mM Tris-HCl buffer, pH 8.6, 2% SDS, 30% glycerol and 5mg/ml DTT at 90° C for one minute. This step was followed by equilibration and alkylation at room temperature in a solution containing 50 mM Tris-HCl buffer, pH 8.6, 6M urea, 2% SDS, 30% glycerol, and 20mg/ml iodoacetamide for 10 minutes.

2.7. Separation of proteins in the second dimension (SDS-PAGE)

After IEF separation, reduction and alkylation steps, the proteins were further separated on a 10% SDS- polyacrylamide gel. The IPG strip containing isoelectrically focused proteins was directly loaded on the top of the separating gel. Electrophoresis was carried out in the dark (to reduce exposure of fluorophores to light) for 18 hours at a constant voltage of 40 volts.

2.8. Gel Imaging and Analysis

The separated proteins labeled with Cy3 and Cy5 fluorophores were detected using 2920 2D-Master Imager (Amersham Biosciences). The Cy3 (primary) and Cy5 (secondary) gel images were then processed using the DeCyder DIA (Differential In-Gel Analysis) software (Amersham Biosciences). This software detects protein spots (spots migrating to the same position on the SDS gel) and quantifies the spot volumes for each image and expresses these volumes as ratios. This ratio can then be used for protein abundance comparisons between the primary Cy3 labeled (−)HU and Cy5 (+)HU SS RBC membrane samples. DeCyder BVA (Biological Variation Analysis) software was used for matching multiple images from different gels for identification of protein spots that are consistently changing in abundance. Statistical analysis was performed on all the protein spots that exhibited ≥ ± 2.5-fold change in protein content at least in one DIGE experiment. Protein spots with statistically significant change in protein content were chosen for identification by mass spectrometry.

2.9. Statistical Analysis

In this study, a pooled membrane sample of 5 SS patients who did not receive hydroxyurea (Cy3 labeled) served as the control group. The treatment group consisted of 5 sickle cell membrane samples from SS patients who received HU (Cy5 labeled). Each individual sickle cell sample from the treatment group was compared with a portion of the pooled control. The data consisted of fold-changes (HU treated/untreated or Cy5/Cy3) in volumes of protein spots in n = 5 pairs of samples. Not every protein spot was observed in every patient. So we only

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analyzed those spots that were observed in at least three pairs of samples. This resulted in data on 89 spots.

The goal of statistical analysis of these data was to discover protein spots whose mean ratios (HU treated/untreated) differed significantly from one. The fold-changes were converted into ratios and then natural log of the ratios was taken to normalize the data. Since the normality assumption was appropriate for these data, we used the two-sided t-test to compute the p-value for testing the null hypothesis that the distribution of log-ratios on each spot had a mean of zero. The p-value for each individual spot was adjusted to account for multiple testing using the false discovery rate (FDR) method [11]. A cutoff of 0.11 was used for the FDR to assess significance of results. This means that 11% of the spots declared to be significant are expected to be false positives. The statistical analysis was performed using the statistical software R [12].

2.10. Protein identification by tandem mass spectrometry and database search

The selected protein spots were excised from Sypro Ruby- stained gels using an Ettan Spot Picker (Amersham Biosciences). The spots were digested using an in-gel trypsin digestion kit (Pierce). The identity of the tryptic peptides generated out of each excised protein spot was determined using LC/MS/MS (LC-Liquid Chromatography and MS-Mass Spectrometry) and database search. The LC/MS/MS was performed on a HPLC system connected to a LCQ XP ion trap mass spectrometer with a nanospray ionization source (ThermoFinnigan). The parameters for the HPLC system were as described by Kakhniashvili et al. [10]. The database used for tryptic peptide search was NCBI non- redundant protein sequence database (human.nr.fasta, 2007) using the Sequest algorithm. The criteria used to confirm the identity of a certain protein was as followed in our laboratory: 1) Xcorr (X Correlation) of at least 1.5, 2.0 and 2.5 for singly, doubly and triply charged peptides respectively and 2) dCn (delta Correlation) of at least 0.1 regardless of charge state [10].

2.11. Immunoblot analysis of protein p55

Immunoblot analysis was performed to assess the levels of protein p55 in SS patients undergoing or not undergoing HU therapy. Following 2D-electrophoresis, proteins were electroblotted onto nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM glycine, 0.005% (w/v) SDS, 20% (v/v) methanol) overnight at 4° C and 25 V. Membranes were blocked for 1 hour with 5% milk blocking agent dissolved in 1X PBST (10mM $NaPO_4$, pH 7.4, 150mM NaCl, 0.05 % Tween 20) and incubated in anti-p55 primary antibody (Santa Cruz Biotechnology, Inc.) overnight at 4 °C followed by four washes of PBST for 5 minutes each. Immnoreactive spots were detected by incubating the membranes for 1 hour in horseradish peroxidase conjugated secondary antibody (Amersham Biosciences). After four PBST washes of 5 minutes each, membranes were developed using ECL plus as the chemiluminiscent system (Amersham Biosciences). Densitometric analysis was performed on the spots using an AlphaImager densitometer (Alpha Innotech).

3. Results

Clinical parameters of SS subjects on HU therapy

Table 1 represents the clinical data of SS patients on HU therapy. All five SS patients in the treated group received high doses of HU ranging from 30 to 38 mg/kg/day for duration of 4 months to 5 years. Based on studies performed by Charache et al. [13], an average HU dose of 35mg/kg/day corresponds to a plasma HU concentration of 400μM. All the SS patients on HU therapy showed a marked decrease in vaso-occlusive episodes and acute chest syndrome with the HU doses received at the time when blood was collected. The HU-treated patients had overall event rates of 0/year while on HU.

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Table 2 represents clinical data from five patients in the control group who never received HU, their ages ranged from 10–50 years, and they suffered at least one pain episode in the preceding year. Patients from both the groups did not receive blood transfusion in the preceding four months. It should be noted that though patients 1175 and 1176 in the control group showed high HbF levels (19.4% and 21.5% respectively), their corresponding MCVs, frequency of pain events and ACS do not indicate any added advantage due to the high HbF levels. The overall MCV values in the treatment group are higher than those in the control group indicative of improved hydration status of sickle RBCs after HU therapy. Similarly the overall frequency of pain events as well as acute chest syndrome is lower in the treatment group as compared to the control group suggesting clinical improvement after HU therapy. There was no information available on HbF levels from patients 1178 and 1179 probably because HbF levels are not frequently monitored in SS patients not on HU therapy.

Statistically significant proteomic changes in SS patients on HU therapy

Our experimental design (Fig. 1) for *in vivo* proteomic analysis consisted of a Cy 3 labeled pooled sickle RBC membrane reference control sample obtained from five SS patients not on HU therapy. This was compared against individual Cy 5 labeled sickle RBC membrane samples obtained from five SS patients on HU therapy. Using a False Discovery Rate (FDR) of 11%, a total of 37 spots exhibited significant change after *in vivo* HU administration. The mean ratio was less than one in only two of these spots, and in the rest, it was more than one. The identity of 32 spots was established using tandem mass spectrometry. Fig. 2 represents a Sypro-Ruby stained 2D gel with the positions of the protein spots of interest marked. Table 3 lists the proteins that demonstrated significant alterations in response to HU therapy.

The altered proteins belonged to four major groups: RBC membrane skeletal proteins, glycolytic enzymes, protein repair participants and protein degradation components. Proteins identified as RBC membrane skeletal components and showing increased expression were protein band 3, ankyrin, protein 4.1, palmitoylated membrane protein (p55), actin, tropomodulin and stomatin. Enzymes participating in glycolysis: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose bisphosphate aldolase were also identified as significantly increasing after HU treatment. As seen in Table 3, it was of common occurrence for a protein to appear in multiple spots, each spot showing the same direction of change. For example, glyceraldehyde-3-phosphate dehydrogenase was identified in spots 20 through 30 with all spots demonstrating a significant increase suggesting HU-induced increase in expression of the protein during erythropoiesis. Of these proteins, p55 was the only common HU target identified in our present *in vivo* and previous *in vitro* analyses of HU- induced changes [7]. The only significant decrease was observed in chaperonin containing TCP1 subunit 2, a component of a large multiprotein TCP1 ring complex involved in the proper folding of actin and tubulin in an ATP-dependent way [14]. A marked decrease in this protein folding/repair component may suggest the failure of the anti-oxidant system to protect against HU-induced oxidative stress at high *in vivo* HU doses of 400μM or above.

Immunoblot analysis of palmitoylated membrane protein 55 (p55)

Our 2D-DIGE and mass spectrometry based proteomic approach identified a significant increase in palmitoylated membrane protein 55 (p55) in patients on HU therapy. In order to confirm these findings, we assessed the levels of p55 from SS RBC membranes prepared from representative blood samples from the control and treatment group. The results obtained from western blot analysis confirmed increased levels of p55 after *in vivo* administration of HU (Fig. 3). Patient 1180 (represented as +(HU)1 in lane 2) received 30mg/kg/day of HU for four years and showed a 5-fold increase in p55 levels as compared to the −(HU) sample from the untreated group (lane 1). Patient 1185 (represented as + (HU)2 in lane 3) received 30mg/mg/day of HU

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for four years, 8 months and demonstrated a 10-fold increase in p55 levels as compared to the −(HU) sample from the untreated group (lane 1).

4. Discussion

Though the *in vivo* effects of HU on the status of RBC dehydration [4], adhesion to endothelium [5], deformability [6] and NO production [15] have been studied, very few protein targets involved in these pathways have been identified. The goal of the present study was to identify protein markers of HU therapy *in vivo.* Since, in the *in vivo* system, the main target of HU is the bone marrow, protein changes at the level of protein expression as well as post-translational modification can be analyzed. Hence these changes are a reflection of changes throughout erythropoiesis as well as after RBC maturation (enucleation). In our previous *in vitro* protein profiling studies, we analyzed HU-mediated quantitative protein changes in membranes of mature, enucleated sickle RBCs exposed to increasing HU concentrations. Due to lack of new protein synthesis in these cells, we mainly anticipated identifying post-translational protein modifications caused by HU. We were successful in demonstrating increased tyrosine phosphorylation of catalase after *in vitro* exposure to low concentrations of HU (50 and 100μM). The current study focused on *in vivo* proteomic analysis of sickle RBC membranes obtained from patients receiving an average dose of 400μM HU. This higher HU concentration achieved *in vivo*, caused a significant increase in RBC membrane skeletal components as well as glycolytic enzymes and a significant decrease in a few protein repair and degradation components. Ankyrin, protein 4.1, β actin, anion exchanger Band 3, palmitoylated membrane protein (p55) and stomatin were the major RBC membrane skeletal components that were identified as targets of HU therapy. Of these proteins, 4.1 and actin are members of the RBC membrane skeleton that covers the cytoplasmic surface of the RBC plasma membrane. The 'spectrin-actin-protein 4.1 ternary complex' is responsible for regulating the elasticity and flexibility of the RBC. The membrane skeleton is attached to the plasma membrane through the binding of protein 4.1 to the transmembrane protein glycophorin C and also through the binding of ankyrin to β spectrin and transmembrane protein band 3 [16]. Previous studies reported improved rheological properties of the sickle RBC membrane in some patients receiving HU therapy [6]. A study undertaken to measure rigidity index (measure of cell rigidity) and elastic sheer modulus (measure of membrane deformability) demonstrated better values after HU therapy [17]. Oxidation of actin [18] and protein 4.1 [19] has been directly observed in the erythrocyte membrane skeleton of patients with SCD. Our laboratory demonstrated that a locked RBC membrane skeleton, as a result of oxidatively damaged actin and spectrin, forms the molecular basis of the abnormal sickle shape [20,21]. Thus, the increased content of crucial ternary complex proteins like protein 4.1 and actin could signify beneficial effects of HU therapy and is likely to have important implications in regulating RBC shape and flexibility in the course of the disease. Also, HU mediated increase in glycolytic enzymes G3PDH and fructose bisphosphate aldolase could satisfy the increased demand for ATP synthesis under conditions of oxidative stress.

Of all the HU-mediated protein changes, the marked increase in erythrocyte membrane protein p55 may signify an important consequence of HU therapy as it was the only common HU target identified in our *in vitro* and *in vivo* analyses of HU induced changes. The increased levels of p55 were further confirmed through immunoblot analysis. P55 is a peripheral membrane protein, most abundantly palmitoylated within human RBCs and constitutively expressed through erythropoiesis [22]. Interestingly, the primary structure of p55 consists of the PDZ domain, the SH3 domain, the tyrosine phosphorylation domain and carboxy-terminal guanylate kinase-like domain. Presence of the N-terminal SH3 domain makes it an important candidate protein in signal transduction [23]. It is suggested that p55 binds in precise proportions to the protein 4.1-glycophorin C complex, linking the plasma membrane to the RBC membrane skeleton and maintaining the stability and mechanical properties of the RBC plasma membrane

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[24]. Considering the possible roles of protein p55 in the RBC membrane skeletal network, and in the formation of a ternary complex with protein 4.1 and glycophorin C, the significant increase in p55 levels at low and high HU doses may represent a key response to HU therapy.

A significant decrease in chaperonin TCP1 subunit 1 and 26S proteasomal subunit α type 4 suggested increased oxidative stress in SS patients on high HU doses. Anti-oxidant enzymes catalase and thioredoxin peroxidase demonstrated a decrease by more than 2.5 fold in three SS patients out of the five under study; however, these changes were not statistically significant.

It would be interesting to note that patients 1180 and P1185 did not show substantial increases in HbF levels (9.5% and 6% respectively) characteristic of SS patients on HU therapy. However, these patients showed excellent clinical improvement after HU therapy and hence were included in the study. Also, we understand that there is variation in the treatment regimen and HbF responses within the treatment group. However, it has been demonstrated in previous literature that HU induced changes like decreased adhesion of sickle erythrocytes to laminin and thrombospondin are observed 1–2 months after initiating therapy and these changes precede substantial increases in HbF [5]. Hence it is possible that HU-mediated proteomic changes observed in our study are independent of HbF levels and reflect immediate (after few months of treatment) as well as long-term responses to HU (after few years of treatment).

A limitation of our study is that the number of patients used is small (5 patients in each of treatment and control groups). Certainly our findings would need to be confirmed in a large study consisting of groups of patients that are comparable except for the HU treatment.

To date 751 proteins have been identified and their interactome network was published by Goodman et al [25]. It will be interesting to determine which HU dependent protein changes are at nodes that represent articulation points within the RBC interactome network. A more recent review with up-to-date coverage of the RBC proteome, as well as the proteome of other blood cells, has recently appeared (26).

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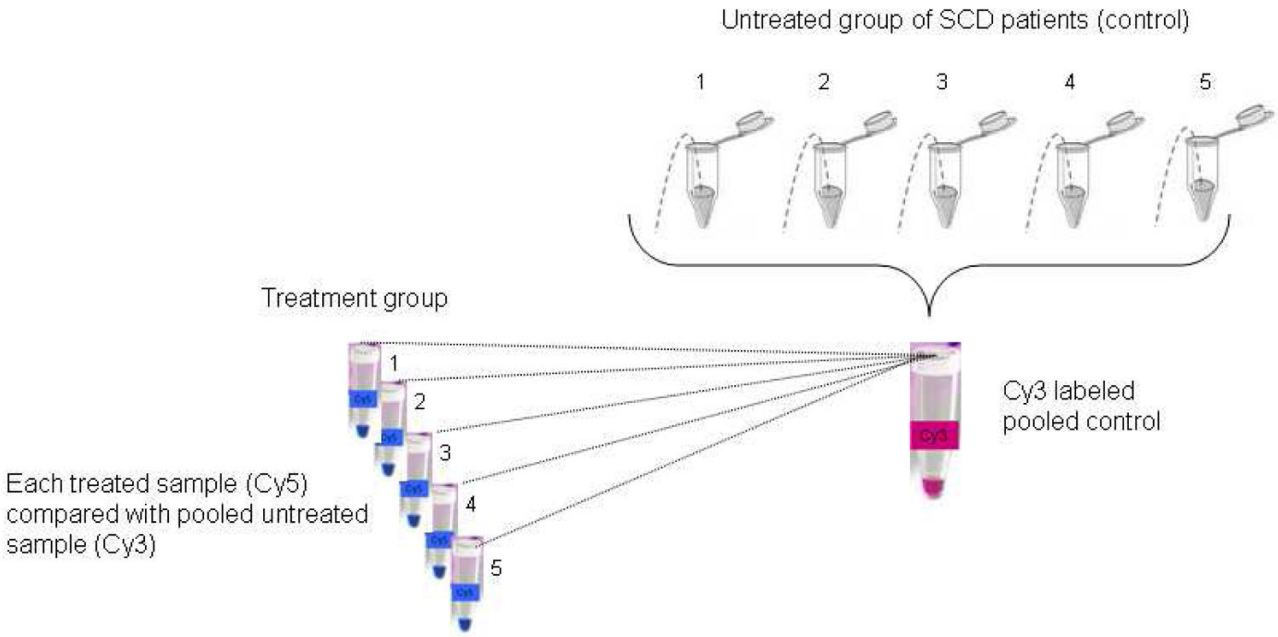

Fig. 1.

Experimental design for *in vivo* proteomic analysis of −/+ HU sickle RBC membranes-RBC membrane samples (100 μg each) obtained from reticulocyte free, mature RBCs of five SS subjects not on HU were pooled and labeled with Cy3 fluorophore. This pooled control was compared against individual Cy 5 labeled sickle RBC membrane samples (100μg) obtained from reticulocyte free, mature RBCs of five SS patients on HU therapy.

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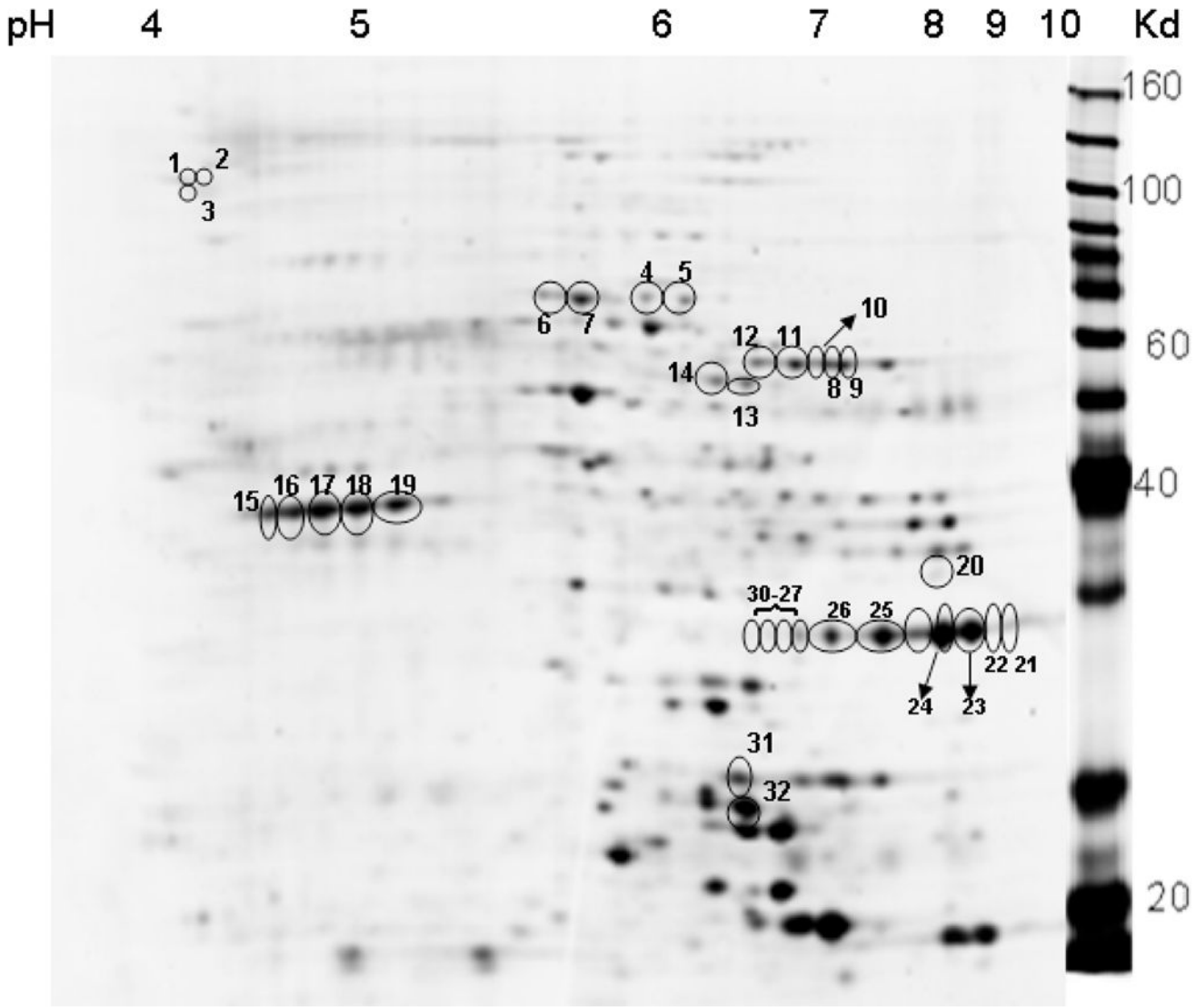

Fig. 2.

A Sypro-Ruby stained representative 2D gel showing positions of SS RBC membrane proteins that showed a statistically significant change in patients undergoing HU treatment ($p < 0.1$). The marked protein spots were selected for protein identification by tandem mass spectrometry. The molecular weight marker lane is indicated on the right and the pH gradient used for isoelectric focusing is indicated on the top, determining the 2D position of any given protein spot based on its pI and molecular weight.

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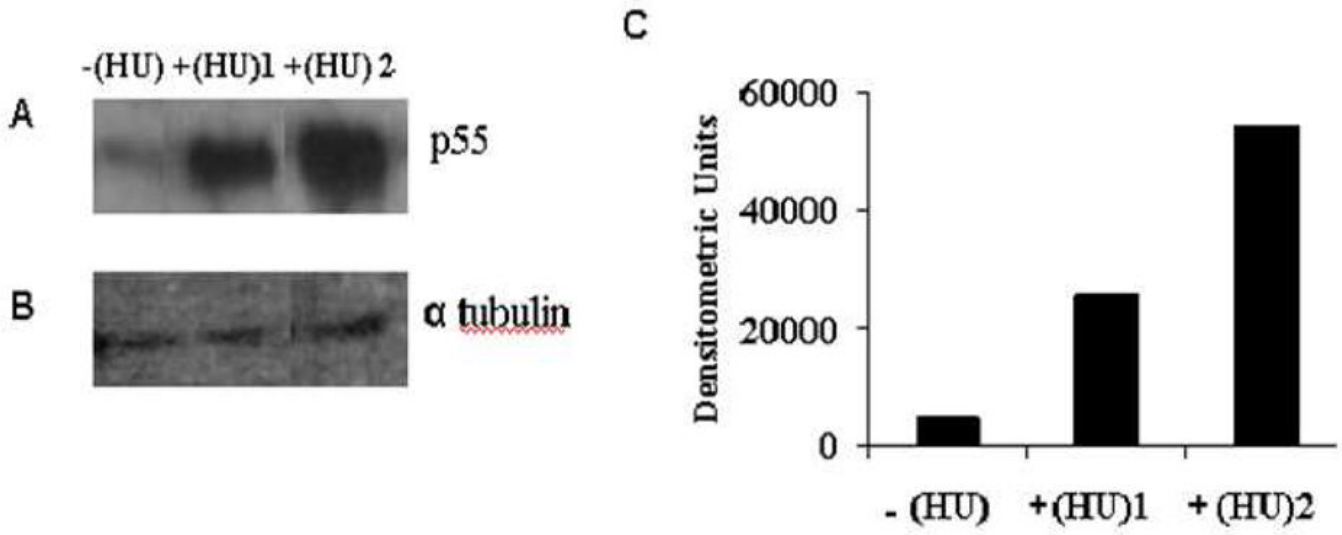

Fig. 3.

Western blot to confirm increased levels of palmitoylated membrane protein (p55) in SS RBC membranes from representative SS patients on HU therapy. Panel A Lane1 - SS RBC membrane sample from the untreated group of SS patients represented as −(HU). Lane 2 and 3 - SS RBC membrane samples from two SS different patients, +(HU)1 and +(HU)2 in the treatment group. Panel B. α tubulin loaded as internal control. Panel C. Densitometric analysis of band intensities. +(HU)1 and +(HU)2 samples showed a 5 fold and 10 fold increase in p55 levels respectively as compared to −(HU) sample.

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Table 1

Clinical data of SS patients on HU therapy

Patient ID	Age	Gender	Hgb	MCV	Retic	HbF	Pain events/year*	ACS events/year*	HU dose mg/kg/day	Duration
1179 (A)	44	F	6.0	117.9	7.6	15.1	3	0	38	7 months
1180 (C)	16	F	9.3	102.6	9.2	9.5	1	2	30	4 years
1181 (A)	23	M	11.7	102.5	12.2	15.8	1	0	38	7 months
1182 (A)	38	M	11.6	122.4	13.1	34.2	5	0	38	4 years
1185 (C)	13	M	9.0	75.2	2.0	6.0	4	0	30	4 years, 8 months

Abbreviations: A-adult, C-child, Hgb-total hemoglobin, MCV-Mean Corpuscular Volume, Retic- Reticulocyte, ACS-Acute Chest Syndrome

*Event rates for pain and ACS are lifetime event rates

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Table 2

Clinical data of SS patients in control group (not on HU therapy)

Patient ID	Age	Hgb (g/dl)	MCV (fl)	Reticulocyte (%)	HbF (%)	Pain events/year	ACS events/year
1175	10	8.6	67.6	4.1	21.4	2	0
1176	43	8.4	76.2	4.2	19.2	4	0
1177	18	9.8	92.0	7.6	4.1	6	4
1178	52	9.6	84.7	10.5	*	3	5
1190	11	7.1	82.3	20.3	*	1	2

* HbF levels of patients 1178 and 1190 were not available at the time of blood collection

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Table 3

Sickle RBC membrane proteins altered *in vivo* in patients on HU therapy

Spot no.	Identified protein	Accession no.	Average volume ratio (+HU/−HU)	Adjusted p value ($\alpha < 0.1$)	Theoretical Mr/pI on 2D gel
1	Solute camel family 4, anion exchanger member 1 (band 3)	4507021	2.45	0.09	101.8/5.10
2	Solute carrier family 4, anion exchanger member 1 (band 3)	4507021	2.45	0.095	52.30/7.22
	Valosin-containing protein	6005942			89.32/5.19
3	N-acylaminoacyl - peptide hydrolase	23510451	2.85	0.095	81.22/5.29
4	Ankyrin 1 isoform 1	105337	2.97	0.002	92.20/6.15
5	Ankyrin 1 isoform 1	105337	3.01	0.065	92.20/6.15
6	Protein 4.1	14916944	2.59	0.085	97.59/4.25
7	Protein 4.1	14916944	2.18	0.095	97.59/4.25
8	Palmitoylated membrane protein (p55)	62898353	2.78	0.065	52.30/7.22
9	Palmitoylated membrane protein (p55)	62898353	2.48	0.067	52.30/7.22
10	Palmitoylated membrane protein (p55)	62898353	2.20	0.095	52.30/7.22
11	Palmitoylated membrane protein (p55)	62898353	2.42	0.095	52.30/7.22
12	Palmitoylated membrane protein (p55)	62898353	2.37	0.095	52.30/7.22
13	Chaperonin containing TCP1, subunit 2	5453603	−2.45	0.095	57.83/6.00
14	β Actin	1419444	3.53	0.065	41.6/5.45
15	Actin	1703156	2.70	0.09	41.77/5.22
16	β Actin	1419444	2.33	0.09	41.6/5.45
17	Tropomodulin	135922	2.80	0.077	40.57/5.03
18	Tropornodulin	135922	2.55	0.095	40.57/5.03
19	Fructose - bisphosphate aldolase	13606	2.03	0.077	39.20/8.01
20	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	4.0	0.042	36.05/8.58
21	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	2.77	0.042	36.05/8.58
22	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	3.17	0.042	36.05/8.58
23	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	3.46	0.043	36.05/8.58
24	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	3.49	0.043	36.05/8.58
25	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	3.82	0.065	36.05/8.58
26	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	3.61	0.065	36.05/8.58
27	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	3.17	0.067	36.05/8.58
28	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	2.61	0.076	36.05/8.58

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Spot no.	Identified protein	Accession no.	Average volume ratio (+HU/−HU)	Adjusted p value (α < 0.1)	Theoretical Mr/pI on 2D gel
29	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	3.53	0.077	36.05/8.58
30	Glyceraldehyde-3-Phosphate dehydrogenase	7669492	1.95	0.095	36.05/8.58
31	Stomatin isform a	38016911	2.49	0.105	31.73/7.90
32	Proteasome subunit alpha type 4	4506185	−4.13	0.105	29.48/7.58