

PROTEIN PROFILING OF SICKLE CELL VERSUS CONTROL RBC CORE MEMBRANE SKELETONS BY ICAT TECHNOLOGY AND TANDEM MASS SPECTROMETRY

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Abstract: A proteomic approach using a cleavable ICAT reagent and nano-LC ESI tandem mass spectrometry was used to perform protein profiling of core RBC membrane skeleton proteins between sickle cell patients (SS) and controls (AA), and determine the efficacy of this technology. The data was validated through Peptide/Protein Prophet and protein ratios were calculated through ASAPratio. Through an ANOVA test, it was determined that there is no significant difference in the mean ratios from control populations (AA1/AA2) and sickle cell versus control populations (AA/SS). The mean ratios were not significantly different from 1.0 in either comparison for the core skeleton proteins (α spectrin, β spectrin, band 4.1 and actin). On the natural-log scale, the variation (standard deviation) of the method was determined to be 14.1% and the variation contributed by the samples was 13.8% which together give a total variation of 19.7% in the ratios.

Key words: Proteomics, Cleavable ICAT, Ion trap mass spectrometry, RBC membrane skeleton, Sickle cell

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Abbreviations used: ASAPratio – automated statistical analysis of protein abundance ratios+; cICAT – cleavable isotope coded affinity tag; CID – collision induced dissociation; 2D DIGE – two dimension differential gel electrophoresis; ESI – electrospray ionization source; ICAT – isotope coded affinity tag; LC – liquid chromatography; RBC – red blood cell; SD – standard deviation; SILAC – stable isotope labeled amino acids in cell culture; WBCs – white blood cells.

INTRODUCTION

Kakhniashvili *et al.* studied the control human RBC proteome through micro liquid chromatography (μ LC) coupled to an ESI tandem mass spectrometer and identified 181 proteins [1]. In order to determine quantitative proteomic changes between control and diseased cells, protein profiling techniques can be used; such as ICAT, 2D DIGE and SILAC [2, 3].

In this study, protein profiling of the core RBC membrane skeleton proteins (α spectrin, β spectrin, band 4.1 and actin) from control and sickle cells using cICAT reagents were analyzed by nano LC ESI tandem mass spectrometry. The principle of the ICAT method is the fact that molecules of identical chemical composition, but which differ isotopically, can be differentiated by their mass difference and the ratio of the mass/charge (m/z) values correspond to the ratio of the analytes [4]. The cleavable ICAT has four functional groups: 1) a protein reactive iodoacetamide group that will react with cysteines, 2) the stable isotopic tag which has a nine ^{12}C chain in the light ICAT or a nine ^{13}C chain in the heavy ICAT, 3) an acid cleavable linker that will reduce the mass contributed by the biotin and thus increase the accuracy in mass determination, and 4) a biotin group for affinity purification [5, 6].

The purpose of this study is the determination of the variance of the cICAT technology as we prepare for future studies in which we will perform protein profiling on RBCs, WBCs and plasma from sickle cell subjects versus controls. We have selected to perform these optimization experiments on core membrane skeleton proteins because of their simple composition. Our results show that the variation, as measured by the SD, of the ICAT method is 14.1%. The additional variation of the core membrane skeleton proteins due to differences in the control populations is negligible. There is no significant difference between the mean ratios of the core membrane proteins from two different controls (AA1/AA2) versus sickle cell (AA/SS); and the mean ratios from control versus sickle cell are equal to one.

MATERIALS AND METHODS

Membrane isolation

Blood was drawn from control (60 ml) and sickle cell donors (40 ml) in Lithium Heparin vacutainers, at the UT Southwestern Comprehensive Sickle Cell Center, and transported at 4°C . To separate plasma from cells, the blood was centrifuged at 290 g for 10 minutes. The RBCs were resuspended in 10 volumes of PBS (10 mM Na_2HPO_4 and 150 mM NaCl) pH 7.6 and sedimented at 2000 g for 10 min at 4°C three times. The cells were lysed in ten volumes of ice cold lysis buffer pH 7.6 (5 mM Na_2HPO_4 and 1mM EDTA) and centrifuged at 3000 g for 10 minutes at 4°C and the supernatant was aspirated. After the first lysate, a white and hard pellet comprised of mononuclear cells was eliminated, and the cells were resuspended in 10 volumes of lysis buffer. This was repeated until the pellet was pale white or slightly pink.

Preparation of core membrane skeletons

The RBC membranes were incubated with the triton extraction buffer (0.6 M KCl, 10 mM Na₂HPO₄, 1mM ATP, 1% Triton X-100, pH 7.6) in a 1:9 volume and incubated for 30 minutes on ice. The solution was centrifuged at 31,000 g for 45 minutes at 4°C. The supernatant was aspirated and the pellet was resuspended in five volumes of 2 M Tris pH 7.4 and incubated at 37°C for 30 minutes. The solution was centrifuged at 35,000 g for 30 minutes at 4°C. The supernatant was dialysed against 50 mM Tris pH 8.5.

ICAT labeling of core membrane skeleton proteins

Membrane skeletal protein concentrations were measured with a Bradford Assay (BioRad). The cICAT protocol provided by Applied Biosystems was followed. It required 100µg of protein from control and sickle cell membrane skeleton proteins. Visualization of cICAT labeling of the sample and its successful trypsinization was demonstrated by SDS PAGE and western blotting utilizing an ECL (Amersham) kit with a streptavidin-horseradish peroxidase conjugate. In sickle cell versus control core skeletal protein profiling, control samples were labeled with the light cICAT and the sickle cell samples with the heavy cICAT. Randomization of this labeling was not needed since it has been demonstrated that the cICAT reagent mass difference does not bias the analysis [7].

Mass spectrometry

The biotin cleaved ICAT labeled peptide pellet was resuspended in 40 µl of 0.1% formic acid, and samples were analyzed by micro-capillary liquid chromatography in line with tandem MS (nLC/MS/MS). A Surveyor high-performance liquid chromatography (HPLC) system was connected to a LCQ DECA XP ion trap mass spectrometer with a nano electrospray ionization source (Thermo Finnigan, San Jose, CA). The nano spray tip (PicoFrit™) has a diameter of 75 µm and a 10 cm C18 column with a pore size of 300 angstrom. The PepFinder kit from Thermo Finnigan allows the production of nano drops to increase the separation of the ions and thus increase the detection to 10 femtomoles. The PepFinder kit incorporates a flow splitter (1:100) and a peptide trap that allows sample loading and desalting at high flow rates, greatly reducing overall analysis times

The Autosampler program and gradient program were utilized. Solution A was composed of 0.1% formic acid and Solution B was composed of acetonitrile and 0.1% formic acid. The flow rate was programmed initially at 100% A at a 10 µl /minute flow rate for 3 minutes. The flow rate was increased to 70 µl/minute for 6.9 minutes at 100% A and the gradient was initiated at 100% A and 0% B. Through the use of the PepFinder Kit, the flow is split in a 1:100 ratio. Thus, the actual flow rate of the sample injected into the mass spectrometer is 0.7 µl /minute. The gradient was increased linearly to 60% B in 60 minutes, then increased to 90% B in 5 minutes and then decreased to 0 % B in 5 minutes and held at 100% A for 10 minutes. The total program time was 101.0 minutes.

Mass spectrum acquisition of the singly, doubly or triply charged ions (+1, +2, +3) was obtained through a “big three” experiment with dynamic exclusion in which the first scan, or “full scan”, detected the m/z spectra from 300 to 2000 m/z. Then, the three most intense ions identified in the “full scan” were isolated and followed by MS/MS CID. The purpose of CID is to fragment the peptides into the y and b series ions in order to determine the peptide sequence. Peptides that were analyzed twice within 30 seconds were excluded in order to detect low intensity peptides. Ion spray conditions were set at a voltage of 1.6 kV, 180 °C, CID energy at 35%.

Bioworks™ Browser v3.1 by Thermofinnigan was used to analyze the RAW data. This software is SEQUEST based [8] with a different user interface. The software uses an algorithm that matches the MS/MS spectra acquired from the mass spectrometer (saved in a RAW file format) and determines a match between those spectra and MS/MS spectra based on a protein database digested virtually with a proteolytic agent. Search parameters were programmed as follows: database, non-redundant (nr) FASTA, restricted to 700-3500 MW human originated peptides; the mass of the atoms considered an average isotopic mix; the enzyme that was used (trypsin) allowing 2 miscleavages. Tolerance and limits for DAT file generation for mass was 1.40 m/z (tolerance of the m/z for an ion), group scan 10, minimum group count 1 and minimum ion count 20. Since this was an cICAT experiment, the static modification of cysteines is programmed with an increase of 227.2613 MW (cICAT tag), and a dynamic search would search for cysteines that present a 9 amu increase (from the nine ¹³C isotope in the cICAT tag). Methionine modification (oxidation: 15.9994 amu) was also programmed in the dynamic search.

The results were filtered through SEQUEST Xcorr score and charge for values equal to or greater than Xcorr 1.5, 2.0 and 2.5 for charge states +1, +2, +3 respectively [1]. Candidate proteins were positively identified when 3 or more peptides matched the protein (unless a biochemical explanation was available) with a Δ Xcorr of greater than 0.1; an Xcorr for charge +1: >2, charge +2: > 2.2 and +3: >3.5. The chromatograms from XPRESS™ were reviewed manually for a correct integration of the data.

To determine the ratio between the light and heavy tagged cICAT peptides XPRESS™ was used. This algorithm integrates the intensity of an ion versus its retention time that corresponds to an cICAT tagged peptide/ion and its \pm 9 amu pair.

The transproteomic pipeline algorithms

The results obtained from the Bioworks™ Browser/Xpress™ showed erratic ratios due to weaknesses of the algorithm to address the noise levels in the MS analysis. Therefore, RAW results from the Mass Spectrometer were reanalyzed through the Transproteomic Pipeline v1.3-4, which uses SEQUEST parameters files [9-12]. Peptide assignments from SEQUEST are validated by Peptide Prophet [10].

Quantitation of the peptides is realized by ASAPratio [11], which determines ratios of control and sickle cell peptides. Protein identification was performed by Protein Prophet [12] and single protein ratios are determined by ASAPratio. Output files are visualized through INTERACT.

Statistical analysis

We analyzed 18 sample pairs and obtained a total of 68 protein ratios. Tab. 1 summarizes their counts at each combination of group (AA1/AA1, AA1/AA2, SS/AA) and the specific proteins. We had two goals for the statistical analysis of these data. The first was to determine the variation in the ratios and assess whether this variation depends on the group or on the protein. The second was to assess whether the mean ratios of the proteins vary from group to group, and discover the proteins in the SS/AA group whose mean ratios are different from one. The mean ratios in the AA1/AA1 and AA1/AA2 groups are expected to be one.

Tab. 1. Number of ratios at each combination of group and specific protein.

Group	Actin	Band 4.1	Spectrin- α	Spectrin- β	Total
AA1/AA1	4	6	6	6	22
AA1/AA2	3	3	3	3	12
SS/AA	7	9	9	9	34
Total	14	18	18	18	68

For the analysis, we assumed an ANOVA model, $Y_{ijk} = I + G_i + S_j + P_k + \varepsilon_{ijk}$ (1), where $Y_{ijk} = \log_e(R_{ijk})$ and R_{ijk} is the ratio of the k -th protein from the j -th sample pair in the i -th group. The natural-log of the ratios was taken to normalize the data. Here the indices $i = 1, 2, 3$ refer to the groups: AA1/AA1, AA1/AA2 and SS/AA, respectively; the indices $j = 1, 2, \dots, 18$ refer to the sample pair numbers; and the indices $k = 1, 2, 3, 4$ refer to the proteins – actin, band 4.1, α spectrin and β spectrin, respectively. In the model (1), I = fixed intercept; G_i = fixed effect of the i -th group; S_j = random effect of the j -th sample pair, S_j follows an independent normal distribution with mean zero and variance σ_s^2 , which we denote as $N(0, \sigma_s^2)$; P_k = fixed effect of the k -th protein; and ε_{ijk} = random error distributed as independent $N(0, \sigma_{\varepsilon_{ijk}}^2)$ variables. We also assumed that the distributions of errors and sample effects are mutually independent. These assumptions are quite standard [13]. Under this model, $I + G_i + P_k = \mu_{ik}$ (say) and $\sigma_s^2 + \sigma_{\varepsilon_{ijk}}^2 = \sigma_{ik}^2$ (say) respectively represent the mean and the variance of

the log-ratios of the k -th protein in the i -th group after averaging over the random sample effects. We tested the hypothesis $\sigma_{11}^2 = \sigma_{12}^2 = \dots = \sigma_{34}^2$ to assess whether the variation in the log-ratios differs significantly from group to group or protein to protein. After this investigation, we compared the mean of the log-ratios. In particular, we tested the hypothesis $\mu_{11} = \mu_{12} = \dots = \mu_{34} = 0$ to determine whether there is a protein in any group whose mean log-ratio is significantly different from zero. We used the likelihood ratio test to compute the p-values for these simultaneous tests [14]. These analyses were performed using the statistical softwares *R* and *SAS*.

To convert means and SD's on the log-ratio scale to the ratio scale, we used the fact that if $Y_{ijk} = \log_e(R_{ijk})$ follows $N(\mu_{ik}, \sigma_{ik}^2)$ distribution, then R_{ijk} follows a log-normal distribution with mean $\exp(\mu_{ik} + \sigma_{ik}^2 / 2)$ and variance $\exp(\sigma_{ik}^2 + 2\mu_{ik})(\exp(\sigma_{ik}^2) - 1)$. The confidence intervals for the mean ratios were computed using the delta method technique [15]. The percentiles of t-distribution with degrees of freedom equal to the number of ratios observed minus one are used as the critical points.

RESULTS

Membrane skeleton isolation

The isolation of membrane skeleton proteins was verified through SDS-PAGE as shown in Fig. 1. The correct labeling and trypsin digestion of the core membrane skeleton proteins were verified through western blotting (results not shown). The major core membrane skeleton proteins were α spectrin, β spectrin, band 4.1 and β actin.

The cICAT technique labels cysteine-containing peptides, which allows a reduction in the complexity of the peptide mixture [16]. Experimentally measured peptide amounts show a reduction from an initial mass of 200 μ g to \sim 30 μ g after cICAT purification. This demonstrates that the complexity in the number of peptides that will be analyzed by the mass spectrometer is reduced dramatically by concentrating on cysteine containing peptides. This allows a reduction in the uncertainty of peptide identification and increased accuracy in relative abundance quantification.

The core membrane skeleton proteins identified through Protein Prophet presented values of 1.0, indicating a high certainty of correct peptide assignments to proteins. Other membrane skeleton proteins were identified with values of 0.95: adducin, ankyrin, band 3, but these substoichiometric proteins were not considered in the analysis. This high certainty of positive identification of the accessory membrane skeleton proteins is due to the sensitivity and resolution of the mass spectrometer.

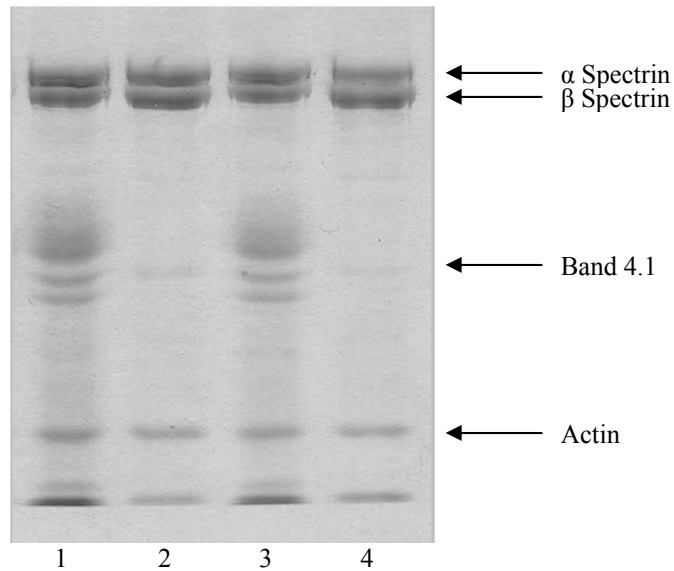


Fig. 1. Membrane skeleton protein preparation analyzed by identified by SDS-PAGE Lane 1: SS RBC membrane protein, lane 2: SS membrane skeleton proteins, lane 3: control (AA) membrane protein, lane 4: AA membrane skeleton proteins. Lanes 2 and 4 are typical of those samples labeled with cICAT reagents.

The variation in the method

The variation of the cICAT method was evaluated by dividing a control sample in two equal aliquots (AA1/AA1) with each part labeled with either the Light or the Heavy cICAT. The mean for each core membrane skeletal protein is not significantly different from 1.0 (Tab. 2 and Fig. 2). From the ANOVA model, the total variation in log ratios is 19.7 %, which when converted to the ratio scale is 18-20%. In Tab. 2 the values determined from the ANOVA model are presented.

Tab. 2. Summary statistics for proteins in AA1/AA1 group.

	n	Mean ratio	95% CI of Mean	SD of ratio	p- value
α Spectrin	6	0.89	[0.75,1.07]	0.18	0.12
β Spectrin	6	0.95	[0.79,1.14]	0.19	0.36
Band 4.1	6	1.01	[0.85, 1.21]	0.20	0.94
β Actin	4	0.90	[0.73,1.13]	0.18	0.18

The mean ratios of the skeletal proteins and the 95% confidence interval are shown in Fig.2. The mean ratio for each of the proteins is not significantly different from 1.0 and the variation is similar for each core skeletal protein.

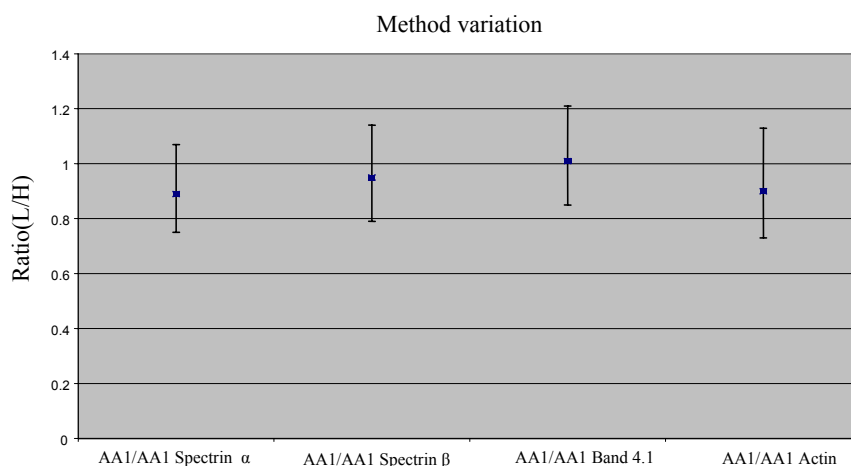


Fig. 2. Ninety five percent confidence intervals for the mean ratio of the core skeletal proteins. Squares indicate mean ratio and the 95% confidence limit for each ratio is presented.

Control (AA) versus sickle cell (SS) ratio analysis

We next determined whether the mean SS to AA core skeletal protein ratios and the mean control population ratios (AA1 versus AA2) were significantly different. The control population studies represent comparisons of RBC membrane skeleton protein samples from pairs of AA subjects, labeled either with the light or the heavy cICAT. The sickle cell versus control ratios were determined by a control sample labeled with the light reagent and the sickle cell sample labeled with the heavy reagent. The protein ratios from control populations can be compared to control versus sickle cell protein ratios since both contain the variation contributed by the method and the variation contributed by the difference between samples.

The values of the mean ratios of α spectrin, β spectrin, band 4.1 and β actin in SS versus AA are not significantly different ($p = 0.10$) from their ratios in control (AA1) versus control (AA2) protein profiling. The mean ratios for each of the membrane skeleton proteins of SS versus AA and AA1 versus AA2 are not significantly different from 1.0 and the standard deviations of the ratios are comparable. The values determined from the ANOVA model are shown in Tab. 3. In Fig. 3, the 95% confidence intervals for the mean ratios of SS versus AA skeletal proteins and the AA1 versus AA2 mean protein ratios are compared. The wider interval in the AA1/AA2 is due to smaller sample size ($N=3$) compared to the SS sample size ($N=9$). However, even if N were increased in the AA samples, the conclusion that the mean ratio is equal to one is not expected to change.

The variation contributed by the samples is 13.8% in log ratios, while the variation contributed by the method is 14.1%. Thus, the total variation in log-ratios is $\sqrt{(0.138^2 + 0.141^2)} = 0.197$ or $\sim 20\%$. When converted to the ratio scale,

the values vary slightly since the estimated means are not all the same. The converted values for the variation are 18 to 22% for the core skeletal proteins. The variability in the log-ratios does not differ significantly (p -value = 0.72) between groups (AA1/AA1, AA1/AA2, SS/AA) or proteins (α spectrin, β spectrin, band 4.1 and β actin). Thus, the contribution to the total variation from the different control individuals is negligible.

Tab. 3. Summary statistics for proteins in AA1/AA2 and SS/AA groups.

	n	Mean ratio	95% CI of mean	SD of ratio	p- value
AA1/AA2 α Spectrin	3	0.96	[0.64,1.44]	0.19	0.59
AA/SS α Spectrin	9	0.97	[0.84,1.11]	0.19	0.39
AA1/AA2 β Spectrin	3	1.02	[0.68,1.53]	0.20	0.99
AA/SS β Spectrin	9	1.03	[0.9,1.18]	0.20	0.90
AA1/AA2 Band 4.1	3	1.09	[0.73,1.63]	0.22	0.55
AA/SS Band 4.1	9	1.10	[0.96,1.26]	0.22	0.26
AA1/AA2 β Actin	3	0.96	[0.64,1.45]	0.19	0.61
AA/SS β Actin	8	0.97	[0.83,1.13]	0.19	0.44

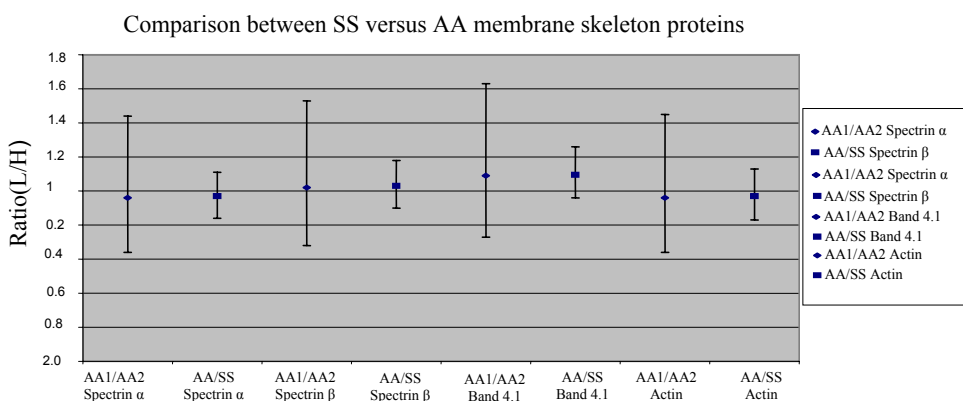


Fig. 3. Protein Profiling of AA versus SS RBC core membrane skeletal proteins and control population (AA1/AA2) proteins.

DISCUSSION

We have established the efficacy of the cICAT labeling technique in protein profiling of human erythrocyte membrane skeleton proteins. We determine that there is no significant difference between the AA versus SS and AA1 versus

AA2 ratios, where the mean ratio value is not significantly different from 1. The method variation (14.1%) and the variation of the sample population (13.8%) summed together determine that the total variation in the ratios is ~20%. This suggests that changes in ratios more than 2 standard deviations i.e. 40% may be statistically significant. Molloy *et al.* [7] treated *E. coli* with Triclosan to inhibit the expression of Fab I, which is involved in fatty acid synthesis, and determined a variation on average of 22.6% in their treated/control ratios.

The use of the second generation acid cleavable $^{12}\text{C}/^{13}\text{C}$ cysteine labeling cICAT reagents addresses the slight delay of the heavy and light cICAT tagged peptides eluting from a C18 reverse phase column by removing biotin prior to LC MS/MS [6, 17-19]. The cICAT technology was designed to address the documented weaknesses of 2D SDS PAGE for protein separation [20-23] such as precipitation of hydrophobic proteins (GRAVY>0.3) and heavily glycosylated proteins, inability to resolve proteins with very small (<10 kDa) or very large (>150 kDa) molecular weights; inability to resolve proteins with very high or very low isoelectric point; and a limited dynamic range. Using an n-LC ion trap mass spectrometer also contributes to increased confidence in peptide identifications due to the ability to determine peptide sequences compared to TOF mass spectrometers that base identification upon mass values for peptides.

Although 2D SDS PAGE has the aforementioned weaknesses, it still provides information on protein isoforms and posttranslational modifications that are not as easily identified when the ICAT protocol is coupled with tandem mass spectrometry. Kakhniashvili *et al.* [24] performed a proteomic study of human control versus sickle cell membrane proteins using a 2D DIGE and n-LC tandem mass spectrometer. In this study, it was determined that 5 protein families: 1) actin accessory proteins, 2) protein repair proteins, 3) lipid rafts, 4) protein turnover components, and 5) scavengers of oxygen radicals changed in excess of 2.5 fold when sickle cell RBC membrane proteins were compared to controls. Protein 4.1, a member of actin accessory protein group presented, in 11 protein spots. These were interpreted as different postrationally modified forms of 4.1 or alternate spliceforms. Several of the 4.1 spots were increased in SS membranes while one decreased by > 2.5 fold. Our results of no change in ratios of band 4.1 in SS/AA, with the cICAT, method indicates that there is no change in the total amount of band 4.1. These results demonstrate the complementarity of the two methods.

Protein profiling through cICAT technology is able to measure ratios of total protein 4.1 in SS versus AA sample with great precision (~20% variation in the technique). The 2D DIGE technology is able to determine ratios of specifically modified forms of 4.1 in SS versus AA samples but with a lower precision (2.5 fold changes). Because the cICAT technology focuses on cysteine containing peptides, it will give very precise ratios but miss many post translational changes. Because of the separate specific strengths of the cICAT MS/MS technology and 2D DIGE MS/MS technology, we apply both techniques to RBC protein profiling studies performed in our laboratory.

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