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Proteome Analysis of the Triton-Insoluble Erythrocyte Membrane Skeleton

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Abstract

Erythrocyte shape and membrane integrity is imparted by the membrane skeleton, which can be isolated as a Triton X-100 insoluble structure that retains the biconcave shape of intact erythrocytes, indicating isolation of essentially intact membrane skeletons. These erythrocyte “Triton Skeletons” have been studied morphologically and biochemically, but unbiased proteome analysis of this substructure of the membrane has not been reported. In this study, different extraction buffers and in-depth proteome analyses were used to more fully define the protein composition of this functionally critical macromolecular complex. As expected, the major, well-characterized membrane skeleton proteins and their associated membrane anchors were recovered in good yield. But surprisingly, a substantial number of additional proteins that are not considered in erythrocyte membrane skeleton models were recovered in high yields, including myosin-9, lipid raft proteins (stomatin, flotillin1 and 2), multiple chaperone proteins (HSPs, protein disulfide isomerase and calnexin), and several other proteins. These results show the membrane skeleton is substantially more complex than previous biochemical studies indicated, and it apparently has localized regions with unique protein compositions and functions. This comprehensive catalog of the membrane skeleton should lead to new insights into erythrocyte membrane biology and pathogenic mutations that perturb membrane stability.

Graphical abstract

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Keywords

Erythrocytes; membrane skeleton; Triton skeleton; membrane composition; membrane structure; proteomics

Introduction

Most cell types contain a two-dimensional protein network on the cytoplasmic face of the plasma membrane, which is termed the membrane skeleton or membrane cytoskeleton. This protein network plays major roles in cell shape, mechanical properties of the membrane, and protein organization. The membrane skeleton of erythrocytes (also called red cells) was first visualized in electron micrographs of detergent extracted erythrocytes [1] and is the most extensively studied prototype for this critical cell component. A representative cartoon model of the erythrocyte membrane and membrane skeleton based upon many biochemical studies conducted by multiple laboratories over the past four decades is summarized in Figure 1. The membrane skeleton is organized as a polygonal network formed by spectrin tetramers that bridge short actin oligomers with five to seven spectrin tetramers bound per actin oligomer [2, 3]. The spectrin-actin network is coupled to the membrane bilayer by association of spectrin with ankyrin, which is in turn bound to the cytoplasmic domain of Band 3 (anion exchanger-1) [4, 5]. The cytoplasmic domain of Band 3 dimers also associates with Band 4.2 [6]. Additional membrane connections are provided at the spectrin-actin junction by a complex between Protein 4.1, 55 kDa palmitoylated protein (p55), and glycophorin C (GPC) [7]. Several proteins responsible for capping actin filaments and defining the length of actin filaments, as well as stabilizing spectrin-actin complexes, have been localized to the actin oligomers and spectrin-actin junctions by electron microscopy [8, 9]. Protein 4.1 is an important structural and regulatory protein as it stabilizes the spectrin-actin interaction [5, 10]. Dematin was initially identified as an endogenous kinase with actin bundling properties [11] that help anchor the membrane skeleton to the lipid bilayer via the glucose transporter-1 (Glut 1). This linkage is facilitated by adducin [12], a protein that functions similar to Protein 4.1 in modulating spectrin-actin interactions [13]. A non-muscle

isoform of tropomyosin is associated with the sides of actin filaments [14] and probably acts as a molecular ruler that helps define the length of the actin oligomers. Adducin associates with the fast-growing end of actin filaments in a complex that caps the filament and promotes assembly of spectrin as mentioned above [15, 16]. Tropomodulin caps the slow-growing end of actin filaments in a ternary complex involving tropomyosin and actin [17, 18]. Lateral interactions among these proteins constitute the spectrin-based composite structure that is anchored to the bilayer through vertical interactions. Current understanding of the erythrocyte membrane and membrane skeleton is described in greater depth in a recent review [19].

Although the membrane skeleton defines critical erythrocyte membrane properties including cell shape, membrane deformability, and membrane integrity, the mechanisms used to achieve these properties are not well understood. Furthermore, despite prior studies at the biochemical level, there are critical gaps and inaccuracies in our knowledge of the composition of the membrane and membrane skeleton. Specifically, proteome analyses described herein indicate that the composition of the erythrocyte membrane skeleton is incomplete, some reported stoichiometries [20] are probably incorrect, and important protein-protein interactions are missing from typical current membrane models (Figure 1). Even our capacity to reconstitute major macromolecular complexes of the membrane skeleton, such as the complete actin-based junctional complex or Band 3-associated complexes, is largely limited to binary or ternary interactions. Furthermore, the structural basis for producing and maintaining the fundamental biconcave shape of erythrocytes, which contributes to efficient gas exchange in vivo, is still not known.

As noted above, one method used in early studies to image and biochemically characterize erythrocyte membrane skeletons was extraction of membranes with non-ionic detergents, particularly Triton X-100 [21]. Triton X-100 solubilizes and extracts the lipid bilayer and proteins imbedded in the bilayer that are not bound to the membrane skeleton. The insoluble fraction, typically termed Triton Skeletons or Triton shells, retains the original shape of the extracted erythrocyte, which is a biconcave disk under physiological conditions for normal erythrocytes [3, 22–24]. Erythrocyte morphology has been shown to be altered by a wide range of hereditary defects that either directly or indirectly perturb membrane integrity, and this altered morphology is retained in Triton Skeletons from mutant cells [25, 26]. Recently, the native structure of the erythrocyte membrane skeleton was confirmed by cryoelectron tomography using Triton Skeletons [27]. In early studies of erythrocyte membranes, Triton Skeletons were utilized to define the composition of the membrane skeleton using 1D and 2D gels, but surprisingly more recent studies of erythrocyte using proteomic techniques have largely neglected evaluation of the membrane skeleton [28–34]. The one exception is a proteomics study by De Palma and co-workers that employed Triton extraction. However, that study used a low concentration of Triton-X-100 on membranes where the surface had been “shaved” using trypsin [35], which most likely affected the composition of the resulting membrane skeletons. Interestingly, they reported several unusual proteins associated with the erythrocyte membrane cytoskeleton, including subunits of chaperones containing T Complex Protein 1 (TCP1).

In this study, the composition of the normal erythrocyte membrane skeleton was evaluated using an unbiased proteome analysis after extraction of purified erythrocyte membranes using several commonly employed Triton X-100 containing extraction buffers and conditions [3, 23, 36–38]. The Triton insoluble fraction and intact membranes were digested with trypsin using filter-assisted sample processing (FASP) [39] prior to LC-MS/MS analysis. Resulting compositions of intact erythrocyte membranes and Triton Skeletons were quantitatively compared to identify the components of the membrane skeleton. In addition to the expected well-studied membrane skeleton proteins and tightly associated membrane anchoring proteins, a substantial number of additional proteins were consistently recovered with similar yields. These results strongly suggest that the erythrocyte membrane skeleton is substantially more complex than indicated by prior biochemical studies, and the wide range in apparent protein abundances is most likely indicative of microdomains within the membrane that have specialized functions. These new insights should contribute to elucidation of mechanisms by which pathogenic mutations within membrane proteins lead to altered cell shape and destabilized membranes resulting in hemolytic anemias.

Materials & Methods

Materials

Protein concentrating units were purchased from Millipore (Billerica, MA); Triton X-100 was from Sigma (St. Louis, MO); electrophoresis reagents were obtained from Invitrogen (Hercules, CA); sequencing grade trypsin was from Promega (Madison, WI), and western blot ECL reagent was obtained from Pierce Biotechnologies (Bedford, MA). Analytical and Trap UPLC columns were acquired from Waters (Milford, MA). Argonaute-2 rabbit monoclonal antibody#2897 (catalogue number: C34C6) was obtained from Cell Signaling Technology (Danvers, MA).

Sample Collection

Fresh blood samples were collected from healthy volunteers within The Wistar Institute with informed written consent, using protocols approved by the institutional ethical review board.

Preparation of Triton Skeletons

The Triton skeleton isolation procedure is summarized in Figure 2. Human erythrocyte membranes, commonly called “ghosts” or “white ghosts” were prepared as previously described [40], and were subsequently diluted to a 90% hematocrit (v/v) in 20 mM sodium phosphate, pH 7.4. The membranes were then mixed with an equal volume of 5% Triton X-100 in a HEPES-based buffer (~5mg/ml of packed ghosts). HEPES buffers used included: HEPES Buffered Saline (HBS) consisting of 125 mM NaCl, 3.75 mM CaCl₂, 2.5 mM MgCl₂ and 20mM HEPES pH 7.4; HBS without divalent cations (-CaMg); HBS with 600mM NaCl (+NaCl), and HBS with 1M KCl (+KCl). The membrane/Triton mixture was kept at room temperature (RT), which was ~23 °C or on ice (0 °C) for 30 min, and the mixture was then layered over ~30 ml of 10% (w/v) sucrose in the same solution used for initial extraction. The membrane skeletons were pelleted at 12,000 rpm (JA-18 rotor Beckman Coulter, 16,000g) for 20 min, and the supernatant and part of the sucrose cushion were removed. The pellet was mixed with an equal volume (~5 ml) of the initial extraction

buffer and was washed two more times as above. The final Triton skeleton pellet was stored on ice.

Filter Assisted Sample Preparation (FASP)

A modification of the FASP method [39] was used to digest samples with trypsin. Briefly, 200 µg of total protein was dissolved in 4% (w/v) SDS (final concentration) solution and was mixed with 8 M Urea, 0.1 M Tris-Cl, pH 8.5 in a 30 kDa MWCO filter unit and centrifuged at $14,000 \times g$ for 20 min. After reduction and alkylation, modified porcine trypsin (enzyme to protein ratio of 1:50) was added and samples were incubated in a hydrated chamber at 37°C for 16–18 hours with gentle agitation. The trypsinized peptides were eluted with two sequential 50 µl extractions using 50 mM ammonium bicarbonate with 0.1% formic acid (final pH 3) to ensure good recovery of peptides. Digests were stored at –20°C until further use.

LC-MS/MS

LC-MS/MS analysis was carried out using a nano-ACQUITY UPLC (Waters, Milford, MA) that was interfaced with an LTQ-Orbitrap XL (Thermo Scientific, Waltham, MA) mass spectrometer. For each analysis, 2 µl of tryptic digest containing an estimated 1.0 µg of digested peptides was loaded onto a 180 µm × 20 mm trap column packed with 5 µm Symmetry C18 resin (Waters, Milford, MA) using solvent A (0.1% formic acid in Milli-Q water) for 5 min, followed by separation in a 75 µm×250 mm analytical 1.7 µm BEH130 C18 column (Waters, Milford, MA) using an 240 min gradient with solvent B (0.1% formic acid in acetonitrile) [41, 42]. Peptide amounts in digests were estimated based on analysis of total protein measured by the modified Lowry assay (Thermo Scientific, Rockford, IL) and assuming an approximate recovery of 25% after trypsin digestion as indicated by similar analysis of standard protein samples. A 25 min blank gradient was run between each sample injection to minimize peptide carryover. Full scans were carried out from 400 to 2000 m/z with 60,000 resolution. MS2 data were acquired through data-dependent analysis of the top six most intense ions with dynamic exclusion enabled for 60 s, monoisotopic precursor selection enabled, and single charged ions rejected.

Label-free Quantitation with MaxQuant

Raw data was processed through the MaxQuant Software package (Ver. 1.5.2.8). Proteins were identified with the Andromeda search engine within the MaxQuant program using the human UniProt database (Ver. July 2014) that was expanded to include commonly observed contaminants. The search was performed with a fragment ion mass tolerance of 0.5 Da and parent ion tolerance of 20 ppm. The protein and peptide false discovery rates (FDR) were set to 5% and 1% respectively. Data were transferred to Excel software for further processing. Proteins identified by single peptides and obvious contaminants (trypsin, keratins, etc.) were removed resulting in protein and peptide FDR of 2.5% and 0.6%, respectively.

Confocal Microscopy

The procedure for fixing and permeabilization of erythrocytes was adapted from our earlier study [43]. Briefly, fixed and permeabilized erythrocytes were incubated with a rabbit anti-

Ago2 antibody, washed three times, incubated with a goat anti-rabbit antibody conjugated with Alexa Fluor® 488 (Molecular Probes, Eugene, Oregon), washed three times and mounted onto poly-L-lysine coated slides with Aqua-Mount (Thermo-Scientific, Waltham, MA). Images were obtained with a Leica TCS SP5 II scanning laser confocal microscope (Leica Microsystems) equipped with AOBS and HyD detectors.

Western Blot Validation

Anti-Argonaute-2 antibody was obtained from Cell Signalling Technology (Danvers, MA as indicated above, and blots were developed using SuperSignal™ West Femto Maximum Sensitivity Substrate from Pierce (Waltham, MA) as previously described [43].

Results

Triton Skeleton Proteome Composition

Proteome compositions of Triton Skeletons, isolated using conditions comparable to those employed for previously published ultra-structural and biochemical studies [3, 19], were determined using LC-MS/MS of tryptic digests. Interestingly, the compositions of Triton Skeletons on SDS gels closely resembled purified erythrocyte membranes and did not appear to be greatly simplified at this level of resolution (Figure 3). This is because the most abundant membrane-associated proteins, which are responsible for the major bands on SDS gels, are either partially or fully retained in the Triton Skeletons. However, proteome analysis showed Triton Skeletons are greatly simplified compared with ghosts as described below.

For ghost and Triton skeleton proteomes, a peptide FDR of 1% was used in the MaxQuant search, and results were then filtered to remove all proteins identified by a single peptide. This resulted in a total ghost proteome of 440 proteins using this method. We then removed all proteins with less than an average of 10 spectral counts (number of MS/MS spectra associated with a given protein or protein group [44, 45]) in replicate analyses of ghosts. This is because quantitative comparisons using spectral counts are not reliable when the number of spectral counts is low [46]. The resulting list of 76 proteins included most high and medium abundance proteins in the erythrocyte membrane (Supplemental Table 1). This table includes gene names and spectral counts in individual analyses of ghosts, as well as percentage recoveries relative to ghosts for all Triton skeleton conditions described below (calculated separately for each protein in each condition relative to the mean of the ghosts).

A commonly used Triton extraction buffer of HBS buffer and Triton X-100 at room temperature resulted in the recovery of 16 proteins with a yield of at least 50% and a total of 35 proteins with a yield of at least 20% relative to ghosts. These proteins were operationally defined as constituting the membrane skeleton and tightly associated interacting proteins. Spectral counts for each individual proteome analysis were normalized using α -spectrin in order to compare recoveries across samples and conditions. Briefly, in order to compare relative recoveries of proteins across different experiments and different extraction conditions, the spectral counts for α -spectrin in individual datasets were divided by the average spectral counts for α -spectrin across multiple ghost proteomes from separate

experiments and this normalization factor was then applied to spectral counts for all detected proteins in that dataset. This results in equal numbers of spectral counts for α -spectrin in all samples and is based upon the fact that spectrin ($\alpha\beta$)₂ tetramers are the prototypical central component of the membrane skeleton and are only associated with the membrane skeleton. The yields of proteins using several common Triton extraction buffers are summarized in Figure 4. As expected, well-defined membrane skeleton proteins, including α -spectrin, β -spectrin, β -actin, and the actin-associated proteins tropomyosin and tropomodulin, were recovered with yields of at least 70%. Proteins known to link the spectrin-actin network to transmembrane anchors, including ankyrin, Protein 4.1, Protein 4.2, α -adducin, β -adducin, dematin, and 55 kDa palmitoylated protein (p55), exhibited variable recoveries ranging from 38 to 69%. This variability presumably reflects differences in affinity for the spectrin-actin skeleton under the extraction conditions used. Recoveries for known transmembrane anchors ranged from 48% for Band 3 to 23% for Glut1. These lower recoveries presumably reflect the fact that not all of these molecules are in direct contact with the membrane skeleton. In addition, yields of some proteins may be reduced due to partial dissociation of complexes during extraction.

While the results described above are consistent with prior studies, the Triton skeleton proteome analyses also showed a substantial number of additional proteins were recovered with yields similar to the well-characterized membrane skeleton associated proteins. For example, myosin 9 was recovered in similar yield to spectrin and actin, indicating it was tightly and completely associated with the membrane skeleton. Additional peripheral proteins that were recovered with similar yields to the well-known membrane skeleton linker proteins included the chaperone proteins HSP71, HSP70, HSPA5, calnexin, endoplasmic, and protein disulfide isomerase A3 (PDIA3).

A commonly used biochemical method of dissociating the major linker proteins, ankyrin and Protein 4.1, from their transmembrane anchors is extraction of ghosts or spectrin depleted membranes with high ionic strength buffers, i.e. buffers with 0.6 – 1.0 M NaCl or KCl [5, 36, 38, 47–49]. Because these interactions are sensitive to high salt, extraction of intact ghosts with Triton buffers containing high salt are expected to dissociate the transmembrane anchors and other ionic strength sensitive membrane anchors from the Triton Skeletons. To evaluate the effects of high salt on Triton Skeletons, HBS was supplemented with either 0.6 M NaCl or 1.0 M KCl. Both high salt buffers showed similar protein recoveries (Supplemental Table 1) and therefore results of all high salt proteome analyses were averaged. As expected, yields of known anchors such as Band 3, Kell, Rh antigen and Glut1 were greatly decreased in the presence of high salt (Figure 4). However, a substantial number of additional transmembrane proteins showed similar behavior. ERMAP, flotillin-1, flotillin-2, and UDP glucosyl transferase (UGGT1) are particularly noteworthy because their recoveries both at physiological ionic strength and in high salt exceeded recoveries of Band 3, the prototypical membrane skeleton anchor. Other lipid bilayer embedded proteins whose recoveries fell between Band 3 and Glut1 included Band 7 (stomatin), Ras-related protein Rap2b, 1-phosphatidylinositol-4-phosphate 5-kinase (PIP4 kinase), GLI pathogenesis-related 2 (GLIPR2), and acetylcholinesterase (AChE), which is also known as Yt blood group antigen.

The effects of high salt on the association of known peripheral membrane linker proteins with the membrane skeleton were variable. The association of dematin and Protein 4.1 with the membrane skeleton was at least as stable in high salt as in physiological conditions, whereas ankyrin, Protein 4.2 and p55 interactions were extensively destabilized and other proteins were minimally affected. The novel associated peripheral membrane proteins identified in this study showed a similar range of behavior. Specifically, myosin interaction with the membrane skeleton was unaffected or slightly stabilized by high salt, endoplasmic and argonaute-2 interactions were substantially destabilized, and most chaperone proteins were minimally affected.

We also evaluated the effects of reducing the extraction temperature to 0 °C because lower temperatures should slow the dissociation of moderate affinity protein-protein interactions. Consistent with this expectation, protein recoveries of Triton Skeletons at 0 °C were generally increased compared to yields at room temperature (Figure 5). For example, only four proteins were recovered with greater than 80% yield relative to ghosts at room temperature in contrast with 14 proteins recovered at greater than 80% yield using a 0 °C extraction. Interestingly, several proteins that were recovered at less than 20% yield at room temperature, and were therefore not included in Figure 4, were recovered with high yields at the lower temperature. These included glycophorin A, a known membrane skeleton anchor, as well as semaphorin A and the urea transporter, two proteins that were not previously considered to be membrane skeleton anchors. In this regard, it is worth noting that Glycophorin A is typically under-represented by spectral counts in LC-MS/MS analyses of tryptic digests because most tryptic peptides from this protein are either glycosylated or very hydrophobic. However, this under-representation is independent of sample type and should not significantly affect percent recovery in different Triton skeleton preparations relative to ghosts. In addition to the proteins shown in Figure 5, a few proteins that were undetectable or present at very low levels in room temperature extracts were recovered with substantial yields in the low temperature extraction including ABCB6, ABCC4, and Piezo ion channel (Supplemental Table 1).

Finally, the effect of deleting divalent cations from the Triton extraction buffer in a room temperature extraction was evaluated because calcium (Ca^{+2}) and magnesium (Mg^{+2}) are known to be involved in modulating some protein-protein interactions in the membrane, and can have roles in cellular signaling in erythrocytes [50, 51]. Surprisingly, deleting divalent cations actually appeared to stabilize most, but not all, interactions. These results suggest the interactions between these proteins and the membrane skeletal complex are mediated by divalent cations.

Discussion

In previous biochemical studies, Triton Skeletons were often used to characterize the erythrocyte membrane skeleton, but to our knowledge this study is the first comprehensive, unbiased analysis of the composition of the membrane skeleton and associated proteins. Proteome analysis of erythrocyte Triton Skeletons demonstrated that the major, well-characterized membrane skeleton proteins were recovered in good yield, and the associated linker and transmembrane proteins were recovered to varying degrees depending upon the

extraction conditions used. As noted earlier, by normalizing to α -spectrin, we correct for sample-to-sample variations in overall recovery of Triton skeletons using the fact that spectrin is completely within the membrane skeleton (100% recovery relative to ghosts). A protein that is 50% recovered in ghosts implies that either 50% of the molecules of this protein are associated with the membrane skeleton or alternatively 100% of these molecules might be attached to the membrane skeleton but due to moderate affinity interactions under the conditions used, about half these molecules are lost during membrane skeleton isolation. When yields of a single protein vary due to different extraction conditions, this implies that the extraction conditions variably affect affinity of the protein for the membrane skeleton. Interestingly, a number of additional transmembrane and peripheral proteins that have not been previously considered in erythrocyte membrane skeleton models were isolated with the membrane skeletons at levels comparable to or higher than some of the well-characterized components. The lipid raft proteins (Band 7 and the flotillins) may directly interact with the membrane skeleton proteins as suggested previously [52] and could play important roles in erythrocyte membrane biology. Similarly, it is interesting that multiple chaperone proteins are recovered with the membrane skeleton, suggesting that they are likely to play crucial roles in stabilizing specific membrane proteins, possibly by facilitating refolding of proteins after partial unfolding under shear stress. Erythrocyte membranes have to repeatedly withstand high mechanical stresses and deformations as erythrocytes squeeze through capillaries and the spleen, and prior studies showed that shear stress disrupts spectrin tetramer associations and reversibly unfolds spectrin domains [53–56]. It is therefore likely that these chaperones may facilitate refolding of spectrin and possibly other membrane proteins.

The presence of argonaute-2 in the membrane skeleton is another surprising observation that has not been previously reported. Argonaute-2 is primarily involved in the microRNA (miRNA) biogenesis pathway and can modulate miRNA stability in most cell types [57]. However, erythrocytes do not have ribosomes or synthesize proteins, so there is no apparent biological function in the processing of miRNAs nor has an alternative function been reported for argonaute in erythrocytes. In addition, argonaute-2 is usually in the cytoplasm, and the reason for its association with the membrane is not apparent. Considering the abundance of mi-RNAs inside erythrocytes [58], it might play a significant and unknown role in erythrocyte biology. Because this observation is especially surprising, we validated the presence of argonaute-2 in the erythrocyte membrane and membrane skeleton by two independent methods - specifically, confocal microscopy and western blotting (Figure 6). Confocal microscopy showed that argonaute-2 is associated with the membrane in all cells. For visualization purposes, a representative single red cell is shown in Figure 6A. Both methods confirm the proteomics results showing the presence of argonaute-2 predominately associated with the membrane rather than the cytosol. Consistent with the proteomics results (Figure 4), a portion of the membrane bound argonaute-2 remains associated with Triton Skeletons.

An important aspect of developing a structural model of the erythrocyte membrane is the relative stoichiometries of different components. Past studies have focused primarily on the most abundant membrane proteins, and, in most cases where stoichiometries have been estimated, relatively imprecise methods such as densitometry of stained SDS gels or I125

labelling have been used. Despite the potential imprecision of such methods, most of the well-characterized membrane proteins fit models similar to the one shown in Figure 1, which assume the complexes illustrated are spread uniformly throughout the membrane [20, 59]. The current proteomics results strongly suggest that a uniform structural model is not entirely appropriate because copy numbers for most new putative membrane skeleton associated proteins identified here appear to be sub-stoichiometric relative to the canonical structure shown in Figure 1. For example, there are approximately 30,000 actin oligomer-based junctional complexes per erythrocyte, and most estimates of copy numbers for well-characterized membrane proteins are either 30,000 copies per cell or close to a multiple thereof. However, a few proteins in the model are less abundant. For example, Kell has been estimated at 3,000–18,000 copies per cell, and some of our newly identified membrane skeleton associated proteins are similarly low in abundance such as Band 7 and AChE, which have been reported to be present at about 10,000 copies/cell [59]. In addition, we can make rough estimates of abundance using spectral counts per kilodalton of mass for peripheral membrane proteins. Using this method, myosin 9 is estimated to be present at about 6,000 copies per cell, which is far less than one molecule per actin junctional complex. This coupled with the consistently high recovery of myosin using a range of extraction conditions strongly suggests this protein is an important component of the membrane skeleton, but its sub-stoichiometric abundance indicates that it is restricted to microdomains with specialized function. It seems likely that myosin-9 plays a role in producing and maintaining the biconcave shape of the erythrocyte. Maintenance of this shape is critically important for efficient gas exchange across the erythrocyte membrane, and therefore, the specific location, interacting partners and role of myosin-9 in erythrocyte shape and architecture need to be further defined.

Similar to myosin, a number of newly identified transmembrane proteins such as ERMAP, UGGT1 and AChE appear to be sub-stoichiometric relative to the canonical actin-spectrin repetitive structure and may play specialized roles in microdomains with specific functions. Other proteins such as PIP4 kinase and Rap2b are likely to participate in cell signaling events. Figure 7 depicts an update to the model presented in Figure 1 where the newly identified membrane skeleton associated proteins have been added. Locations for most of these proteins within the membrane architecture are currently unknown. The next step is to define key protein-protein interactions so that the locations of these proteins can be more precisely mapped and so that functional studies can be designed. We plan to systematically interrogate protein-protein interactions in intact membranes and membrane skeletons using chemical crosslinking coupled with mass spectrometry analysis.

In summary, our proteome analyses of erythrocyte Triton Skeletons have identified a substantial number of poorly characterized membrane proteins that appear to be either putative members of the membrane skeleton or bind to it and link it to the lipid bilayer. Many of the newly identified components are substoichiometric relative to the canonical repetitive membrane skeleton structure, which suggests that many of these proteins may be associated with microdomains with specialized functions. These results indicate that the erythrocyte membrane skeleton is substantially more complex than previously appreciated. The proteins defined herein provide a context for further systematic analysis of protein-protein interactions within the membrane as well as follow-up functional studies in order to

provide new insights into mechanisms for maintaining erythrocyte membrane shape, size and elasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Yu J, Fischman DA, Steck TL. Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. *J Supramol Struct.* 1973; 1:233–248. [PubMed: 4804838]
2. Liu SC, Derick LH, Palek J. Visualization of the hexagonal lattice in the erythrocyte membrane skeleton. *J Cell Biol.* 1987; 104:527–536. [PubMed: 2434513]
3. Shen BW, Josephs R, Steck TL. Ultrastructure of the Intact Skeleton of the Human-Erythrocyte Membrane. *Journal of Cell Biology.* 1986; 102:997–1006. [PubMed: 2936753]
4. Bennett V, Stenbuck PJ. Identification and Partial-Purification of Ankyrin, the High-Affinity Membrane Attachment Site for Human-Erythrocyte Spectrin. *Journal of Biological Chemistry.* 1979; 254:2533–2541. [PubMed: 372182]
5. Tyler JM, Reinhardt BN, Branton D. Associations of Erythrocyte-Membrane Proteins - Binding of Purified Bands 2.1 and 4.1 to Spectrin. *Journal of Biological Chemistry.* 1980; 255:7034–7039. [PubMed: 6771281]
6. Yu J, Steck TL. Isolation and Characterization of Band-3, Predominant Polypeptide of Human Erythrocyte-Membrane. *Journal of Biological Chemistry.* 1975; 250:9170–9175. [PubMed: 1194279]
7. Marfatia SM, Lue RA, Branton D, Chishti AH. In-Vitro Binding-Studies Suggest a Membrane-Associated Complex between Erythroid P55, Protein-4.1, and Glycophorin-C. *Journal of Biological Chemistry.* 1994; 269:8631–8634. [PubMed: 8132590]
8. Derick LH, Liu SC, Chishti AH, Palek J. Protein Immunolocalization in the Spread Erythrocyte-Membrane Skeleton. *European Journal of Cell Biology.* 1992; 57:317–320. [PubMed: 1387364]
9. Ursitti JA, Fowler VM. Immunolocalization of tropomodulin, tropomyosin and actin in spread human erythrocyte skeletons. *J Cell Sci.* 1994; 107(Pt 6):1633–1639. [PubMed: 7962203]
10. Ungewickell E, Bennett PM, Calvert R, Ohanian V, Gratzner WB. In vitro Formation of a Complex between Cytoskeletal Proteins of the Human-Erythrocyte. *Nature.* 1979; 280:811–814. [PubMed: 471052]
11. Husainchishti A, Faquin W, Wu CC, Branton D. Purification of Erythrocyte Dematin (Protein-4.9) Reveals an Endogenous Protein-Kinase That Modulates Actin-Bundling Activity. *Journal of Biological Chemistry.* 1989; 264:8985–8991. [PubMed: 2722813]
12. Khan AA, Hanada T, Mohseni M, Jeong JJ, Zeng LX, Gaetani M, et al. Dematin and adducin provide a novel link between the spectrin cytoskeleton and human erythrocyte membrane by directly interacting with glucose transporter-1. *Journal of Biological Chemistry.* 2008; 283:14600–14609. [PubMed: 18347014]

13. Koshino I, Mohandas N, Takakuwa Y. Identification of a Novel Role for Dematin in Regulating Red Cell Membrane Function by Modulating Spectrin-Actin Interaction. *Journal of Biological Chemistry*. 2012; 287:35244–35250. [PubMed: 22927433]
14. Fowler VM, Bennett V. Erythrocyte-Membrane Tropomyosin - Purification and Properties. *Journal of Biological Chemistry*. 1984; 259:5978–5989. [PubMed: 6715382]
15. Gardner K, Bennett V. Modulation of Spectrin Actin Assembly by Erythrocyte Adducin. *Nature*. 1987; 328:359–362. [PubMed: 3600811]
16. Kuhlman PA, Hughes CA, Bennett V, Fowler VM. A new function for adducin - Calcium calmodulin-regulated capping of the barbed ends of actin filaments. *Journal of Biological Chemistry*. 1996; 271:7986–7991. [PubMed: 8626479]
17. Fowler VM. Tropomodulin - a Cytoskeletal Protein That Binds to the End of Erythrocyte Tropomyosin and Inhibits Tropomyosin Binding to Actin. *Journal of Cell Biology*. 1990; 111:471–482. [PubMed: 2380244]
18. Weber A, Pennise CR, Babcock GG, Fowler VM. Tropomodulin Caps the Pointed Ends of Actin-Filaments. *Journal of Cell Biology*. 1994; 127:1627–1635. [PubMed: 7798317]
19. Fowler VM. The human erythrocyte plasma membrane: a Rosetta Stone for decoding membrane-cytoskeleton structure. *Curr Top Membr*. 72:39–88. [PubMed: 24210427]
20. Mankelov TJ, Satchwell TJ, Burton NM. Refined views of multi-protein complexes in the erythrocyte membrane. *Blood Cells Molecules and Diseases*. 2012; 49:1–10.
21. Hainfeld JF, Steck TL. The sub-membrane reticulum of the human erythrocyte: a scanning electron microscope study. *J Supramol Struct*. 1977; 6:301–311. [PubMed: 592817]
22. Sheetz MP, Sawyer D. Triton shells of intact erythrocytes. *J Supramol Struct*. 1978; 8:399–412. [PubMed: 723274]
23. Shen BW, Josephs R, Steck TL. Ultrastructure of Unit Fragments of the Skeleton of the Human-Erythrocyte Membrane. *Journal of Cell Biology*. 1984; 99:810–821. [PubMed: 6470041]
24. Matsuzaki F, Sutoh K, Ikai A. Structural unit of the erythrocyte cytoskeleton. Isolation and electron microscopic examination. *Eur J Cell Biol*. 1985; 39:153–160. [PubMed: 4085499]
25. Liu SC, Derick LH, Agre P, Palek J. Alteration of the erythrocyte membrane skeletal ultrastructure in hereditary spherocytosis, hereditary elliptocytosis, and pyropoikilocytosis. *Blood*. 1990; 76:198–205. [PubMed: 2364170]
26. Shinar E, Shalev O, Rachmilewitz EA, Schrier SL. Erythrocyte membrane skeleton abnormalities in severe beta-thalassemia. *Blood*. 1987; 70:158–164. [PubMed: 3593963]
27. Nans A, Mohandas N, Stokes DL. Native ultrastructure of the red cell cytoskeleton by cryo-electron tomography. *Biophys J*. 101:2341–2350. [PubMed: 22098732]
28. Pasini EM, Kirkegaard M, Mortensen P, Lutz HU, Thomas AW, Mann M. In-depth analysis of the membrane and cytosolic proteome of red blood cells. *Blood*. 2006; 108:791–801. [PubMed: 16861337]
29. Roux-Dalvai F, de Peredo AG, Simo C, Guerrier L, Bouyssie D, Zanella A, et al. Extensive Analysis of the Cytoplasmic Proteome of Human Erythrocytes Using the Peptide Ligand Library Technology and Advanced Mass Spectrometry. *Molecular & Cellular Proteomics*. 2008; 7:2254–2269. [PubMed: 18614565]
30. Basu A, Chakrabarti A. Defects in Erythrocyte Membrane Skeletal Architecture. *Biochemical Roles of Eukaryotic Cell Surface Macromolecules*. 2015; 842:41–59.
31. Basu A, Saha S, Karmakar S, Chakravarty S, Banerjee D, Dash BP, et al. 2D DIGE based proteomics study of erythrocyte cytosol in sickle cell disease: Altered proteostasis and oxidative stress. *Proteomics*. 2013; 13:3233–3242. [PubMed: 24030922]
32. Saha S, Ramanathan R, Basu A, Banerjee D, Chakrabarti A. Elevated levels of redox regulators, membrane-bound globin chains, and cytoskeletal protein fragments in hereditary spherocytosis erythrocyte proteome. *European Journal of Haematology*. 2011; 87:259–266. [PubMed: 21575061]
33. Basu A, Chakrabarti A. Hemoglobin interacting proteins and implications of spectrin hemoglobin interaction. *J Proteomics*. 2015

34. Goodman SR, Daescu O, Kakhniashvili DG, Zivanic M. The proteomics and interactomics of human erythrocytes. *Experimental Biology and Medicine*. 2013; 238:509–18. [PubMed: 23856902]
35. De Palma A, Roveri A, Zaccarin M, Benazzi L, Daminelli S, Pantano G, et al. Extraction methods of red blood cell membrane proteins for Multidimensional Protein Identification Technology (MudPIT) analysis. *J Chromatogr A*. 2010; 1217:5328–5336. [PubMed: 20621298]
36. Sheetz MP. Integral membrane protein interaction with Triton cytoskeletons of erythrocytes. *Biochim Biophys Acta*. 1979; 557:122–134. [PubMed: 549630]
37. Vertessy BG, Steck TL. Elasticity of the human red cell membrane skeleton. Effects of temperature and denaturants. *Biophys J*. 1989; 55:255–262. [PubMed: 2713438]
38. Navarro-Prigent MJ, Seguin I, Boivin P, Dhermy D. Study of human erythrocyte membrane protein interactions by selective solubilization of Triton-skeletons. *Biol Cell*. 1995; 83:33–38. [PubMed: 7647706]
39. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nature Methods*. 2009; 6:359–U60. [PubMed: 19377485]
40. Pesciotta EN, Sriswasdi S, Tang HY, Mason PJ, Bessler M, Speicher DW. A label-free proteome analysis strategy for identifying quantitative changes in erythrocyte membranes induced by red cell disorders. *Journal of Proteomics*. 2012; 76:194–202. [PubMed: 22954596]
41. Beer LA, Wang H, Tang HY, Cao Z, Chang-Wong T, Tanyi JL, et al. Identification of multiple novel protein biomarkers shed by human serous ovarian tumors into the blood of immunocompromised mice and verified in patient sera. *PLoS One*. 2013; 8:e60129. [PubMed: 23544127]
42. Tang HY, Beer LA, Tanyi JL, Zhang R, Liu Q, Speicher DW. Protein isoform-specific validation defines multiple chloride intracellular channel and tropomyosin isoforms as serological biomarkers of ovarian cancer. *J Proteomics*. 2013; 89:165–178. [PubMed: 23792823]
43. Pesciotta EN, Sriswasdi S, Tang HY, Speicher DW, Mason PJ, Bessler M. Dysferlin and Other Non-Red Cell Proteins Accumulate in the Red Cell Membrane of Diamond-Blackfan Anemia Patients. *PLoS One*. 2014; 9
44. Lundgren DH, Hwang SI, Wu L, Han DK. Role of spectral counting in quantitative proteomics. *Expert Rev Proteomics*. 2010; 7:39–53. [PubMed: 20121475]
45. Zhu W, Smith JW, Huang CM. Mass spectrometry-based label-free quantitative proteomics. *J Biomed Biotechnol*. 2010; 2010:840518. [PubMed: 19911078]
46. Ahrne E, Molzahn L, Glatter T, Schmidt A. Critical assessment of proteome-wide label-free absolute abundance estimation strategies. *Proteomics*. 2013; 13:2567–2578. [PubMed: 23794183]
47. Leto TL, Marchesi VT. A structural model of human erythrocyte protein 4.1. *J Biol Chem*. 1984; 259:4603–4608. [PubMed: 6707022]
48. Weaver DC, Marchesi VT. The structural basis of ankyrin function. I. Identification of two structural domains. *J Biol Chem*. 1984; 259:6165–6169. [PubMed: 6233273]
49. Tyler JM, Hargreaves WR, Branton D. Purification of two spectrin-binding proteins: biochemical and electron microscopic evidence for site-specific reassociation between spectrin and bands 2.1 and 4.1. *Proc Natl Acad Sci U S A*. 1979; 76:5192–5196. [PubMed: 291934]
50. Andrews DA, Yang L, Low PS. Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells. *Blood*. 2002; 100:3392–3399. [PubMed: 12384442]
51. George A, Pushkaran S, Konstantinidis DG, Koochaki S, Malik P, Mohandas N, et al. Erythrocyte NADPH oxidase activity modulated by Rac GTPases, PKC, and plasma cytokines contributes to oxidative stress in sickle cell disease. *Blood*. 2013; 121:2099–2107. [PubMed: 23349388]
52. Ciana A, Achilli C, Balduini C, Minetti G. On the association of lipid rafts to the spectrin skeleton in human erythrocytes. *Biochimica Et Biophysica Acta-Biomembranes*. 2011; 1808:183–190.
53. An X, Lecomte MC, Chasis JA, Mohandas N, Gratzer W. Shear-response of the spectrin dimertetramer equilibrium in the red blood cell membrane. *J Biol Chem*. 2002; 277:31796–31800. [PubMed: 12105217]
54. Li J, Lykotrafitis G, Dao M, Suresh S. Cytoskeletal dynamics of human erythrocyte. *Proc Natl Acad Sci U S A*. 2007; 104:4937–4942. [PubMed: 17360346]

55. Johnson CP, Tang HY, Carag C, Speicher DW, Discher DE. Forced unfolding of proteins within cells. *Science*. 2007; 317:663–666. [PubMed: 17673662]
56. Law R, Liao G, Harper S, Yang G, Speicher DW, Discher DE. Pathway shifts and thermal softening in temperature-coupled forced unfolding of spectrin domains. *Biophys J*. 2003; 85:3286–3293. [PubMed: 14581229]
57. Cenik ES, Zamore PD. Argonaute proteins. *Curr Biol*. 21:R446–R449. [PubMed: 21683893]
58. Chen SY, Wang Y, Telen MJ, Chi JT. The genomic analysis of erythrocyte microRNA expression in sickle cell diseases. *PLoS One*. 2008; 3:e2360. [PubMed: 18523662]
59. Burton NM, Bruce LJ. Modelling the structure of the red cell membrane. *Biochem Cell Biol*. 89:200–215. [PubMed: 21455271]

Biological Significance

Current models of erythrocyte membranes describe fairly simple homogenous structures that are incomplete. Proteome analysis of the erythrocyte membrane skeleton shows that it is quite complex and includes a substantial number of proteins whose roles and locations in the membrane are not well defined. Further elucidation of interactions involving these proteins and definition of microdomains in the membrane that contain these proteins should yield novel insights into how the membrane skeleton produces the normal biconcave erythrocyte shape and how it is perturbed in pathological conditions that destabilize the membrane.

Highlights

- ❖ Proteome analysis defines a comprehensive erythrocyte Triton skeleton.
- ❖ The erythrocyte membrane skeleton is more complex than current models suggest.
- ❖ Myosin 9, lipid raft proteins, and chaperones are components of membrane skeletons.
- ❖ Substoichiometric membrane skeleton components exist in specialized microdomains.
- ❖ Interactions and functions of important membrane proteins are yet to be defined.

Figure 1. Classical model of the erythrocyte membrane

Schematic representation of the erythrocyte membrane and associated spectrin-actin membrane skeleton structure depicting the two major multi-protein complexes that span the lipid bilayer and anchor the membrane skeleton to the bilayer. As illustrated, there is substantial overlap in the composition of the two major membrane associated complexes. The Ankyrin Complex links the spectrin-actin based membrane skeleton to the lipid bilayer via interaction of ankyrin with β -spectrin. The Protein 4.1 complex anchors the membrane skeleton to the lipid bilayer by association of Protein 4.1 and other linker proteins with a multi-protein complex consisting of short actin filaments, actin-associated proteins and spectrin.

Figure 2. Purification scheme for isolation of Triton Skeletons

Flow diagram summarizing isolation of erythrocyte membrane skeletons using Triton X-100 in HBS buffer. Several different extraction conditions were evaluated and multiple experiments were performed using erythrocytes purified from different donors for most conditions. All extractions utilized a final concentration of 2.5% Triton X-100 and were performed at room temperature except for the condition labeled “0 °C”. Other conditions involved modifications to the HBS buffer as indicated (see Methods for full details).

Figure 3. Preparation of Triton Skeletons using different extraction conditions

Sequential supernatants (S1-3) and pellets (P1-3) were analyzed on 10% Bis-Tris gels using the MOPS buffer system. Sample volumes of 1 μ l for WG and pellet fractions and 7 μ l for supernatant fractions were loaded. All extractions were performed at room temperature with the exception of the extraction labeled as 0 $^{\circ}$ C and all conditions used HBS buffer with a final Triton concentration of 2.5% (see Methods for precise buffer compositions): A) A representative experiment using four different extraction conditions: HBS, 0 $^{\circ}$ C, -Ca/Mg, +NaCl. B) A representative experiment using either HBS or a high ionic strength buffer containing 1M KCl (+KCl).

Figure 4. Protein recoveries in Triton Skeletons using different ionic strength buffers

To compare relative recoveries of proteins across different experiments and different extraction conditions, the spectral counts for α-spectrin in individual datasets were normalized to the average spectral counts for α-spectrin across multiple ghost proteomes from three separate experiments (7 datasets). The 35 major proteins with a recovery of greater than 20% relative to ghosts using HBS buffer/Triton at room temperature (HBS) are shown and represent the average of 8 datasets from three separate experiments (three different donors) using similar conditions. The “High Salt” data represents the combined average of two datasets using NaCl and two datasets using KCl in two separate experiments (two different donors). Error bars show standard deviations.

Figure 5. Protein recoveries in Triton Skeletons using low temperature or buffer without divalent cations

Recoveries of proteins relative to ghost proteomes using HBS at 0°C and HBS with deletion of divalent cations are shown. The values for HBS (at room temperature) shown in Figure 4 are repeated here to facilitate comparisons across all experiments. The HBS/0 °C data and HBS/-CaMg are averages of duplicate analyses (one donor per extraction condition). Error bars show standard deviations. The 40 proteins showing at least a 14% recovery relative to ghosts in the HBS/RT datasets are shown.

Figure 6. Presence of Argonaute-2 protein in erythrocyte membranes and membrane skeletons Proteome analysis results indicating the presence of argonaute-2 were confirmed using (A) Confocal microscopy (in erythrocyte membranes) and (B) Western blotting white ghosts (WG) and associated with Triton skeletons (TS).

Figure 7. New model of the erythrocyte membrane skeleton and associated integral membrane proteins

A substantial number of proteins that are not normally considered to be associated with the membrane skeleton were recovered in Triton skeleton proteomes with yields comparable to the well characterized membrane skeletal proteins and their tightly associated membrane anchors. This indicates these additional proteins are either components of the membrane skeleton or bind to it with high affinity. The new proteins from Figure 4 are indicated by dashed red ovals with red lettering. Both previously known and new proteins are color coded based upon the level of recovery of that protein in the HBS/RT Triton Skeletons relative to ghosts: = >80%, = 60–80%, = 40–60%, = 20–40%, = <20% or not detected. Locations of labels for most new components are arbitrary because specific sites of interaction with the previously known membrane skeleton associated proteins have not been defined.