Journal of Proteomics 128 (2015) 298–305

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Proteome analysis of the triton-insoluble erythrocyte membrane skeleton

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ARTICLE INFO

Article history:
Received 27 May 2015
Received in revised form 24 July 2015
Accepted 5 August 2015
Available online 10 August 2015

Keywords:
Erythrocytes
Membrane skeleton
Triton Skeleton
Membrane composition
Membrane structure
Proteomics

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 that 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.

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.

1. 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 Fig. 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

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http://dx.doi.org/10.1016/j.jprot.2015.08.004
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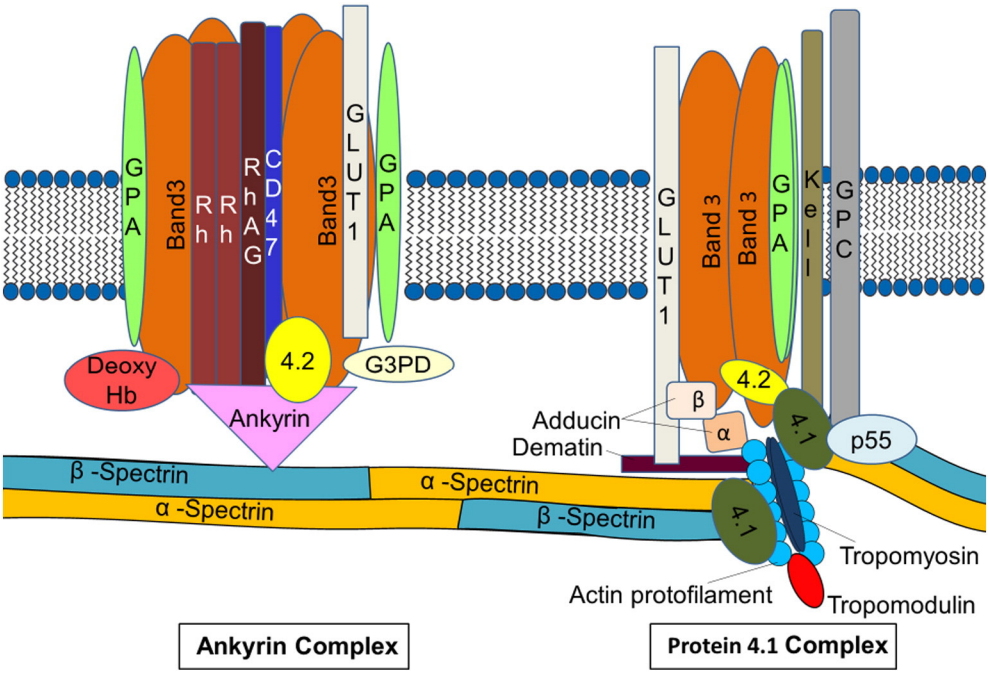

Fig. 1. Classical model of the erythrocyte membrane. Schematic representation of the erythrocyte membrane and associated spectrin–actin membrane skeleton structure depicting the two major multi-protein complexes that span the lipid bilayer and anchor the membrane skeleton to the bilayer. As illustrated, 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.

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 (Fig. 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 coworkers 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