Biochimica et Biophysica Acta xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Stomatin interacts with GLUT1/SLC2A1, band 3/SLC4A1, and aquaporin-1 in human erythrocyte membrane domains

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ARTICLE INFO

Article history:
Received 21 June 2012
Received in revised form 20 October 2012
Accepted 26 November 2012
Available online xxxx

Keywords:
Integral membrane proteins
Lipid rafts
Chemical cross-linking
Protein–protein interaction

ABSTRACT

The widely expressed, homo-oligomeric, lipid raft-associated, monotopic integral membrane protein stomatin and its homologues are known to interact with and modulate various ion channels and transporters. Stomatin is a major protein of the human erythrocyte membrane, where it associates with and modifies the glucose transporter GLUT1; however, previous attempts to purify hetero-oligomeric stomatin complexes for biochemical analysis have failed. Because lateral interactions of membrane proteins may be short-lived and unstable, we have used *in situ* chemical cross-linking of erythrocyte membranes to fix the stomatin complexes for subsequent purification by immunoaffinity chromatography. To further enrich stomatin, we prepared detergent-resistant membranes either before or after cross-linking. Mass spectrometry of the isolated, high molecular, cross-linked stomatin complexes revealed the major interaction partners as glucose transporter-1 (GLUT1), anion exchanger (band 3), and water channel (aquaporin-1). Moreover, ferroportin-1 (SLC40A1), urea transporter-1 (SLC14A1), nucleoside transporter (SLC29A1), the calcium-pump (Ca-ATPase-4), CD47, and flotillins were identified as stomatin-interacting proteins. These findings are in line with the hypothesis that stomatin plays a role as membrane-bound scaffolding protein modulating transport proteins.

1. Introduction

Stomatin, also known as band 7 integral membrane protein or protein 7.2b, is a major erythrocyte membrane protein [1–3] that is missing in red cells of overhydrated hereditary stomatocytosis patients [3]. It is expressed ubiquitously and conserved from archaea to mammals. In humans, there are 5 similar proteins [4–6], while the *C. elegans* genome contains 10 stomatin-like genes, including *mec-2*, *unc-1*, and *unc-24* as best studied [7,8]. The common domain of stomatin-like and related proteins is known as SPFH (stomatin, flotillin, prohibitin, HflC/K)-domain [9,10] or PHB (prohibitin homology)-domain [11]. These SPFH/PHB-proteins may play a role as membrane-bound scaffolding proteins that are associated with other membrane proteins and cortical cytoskeleton [11,12].

Hallmarks of stomatin are the monotopic structure [13], oligomeric nature [14,15], S-palmitoylation [16], and lipid raft-association [15,17,18]. Moreover, stomatin and stomatin-like proteins are cholesterol-binding proteins [4,19]. Most of these features are also characteristic for other SPFH/PHB-domain proteins and for the topologically similar but unrelated caveolins [20]. The crystal structure of an archaeal stomatin core domain revealed a unique, trimeric structure with extending α-helices from each triangular corner that interact with equal α-helices of adjacent trimers to form antiparallel coiled-coils thus explaining the homo-oligomeric nature [21]. In contrast, crystal structures of the mouse stomatin-domain were found to be composed of banana-shaped dimers similar to BAR-domains forming hexagonal structures that are capable of building oligomers [22]. While stomatin and stomatin-like proteins are known to interact with various ion channels modulating their activities [19,22–25], only human stomatin has been shown to associate with the glucose transporter GLUT1 [26–30]. The interaction of stomatin and GLUT1 is also implicated by the loss of function of this complex in erythrocytes of patients with stomatin-deficient cryohydrocytosis [31]. Apparently, stomatin modulates GLUT1 to repress glucose uptake while enhancing dehydroascorbate influx [30]. The molecular mechanism of this modulation has not been investigated yet. Because erythrocyte GLUT1 is only found in mammals that are unable to synthesize vitamin C, it is implicated that the high GLUT1 expression in human erythrocytes may be due to a compensatory mechanism for better utilising ascorbate [30]. This stomatin-dependent mechanism was debated [32,33] and therefore we set out to study the direct physical interaction of these proteins by *in situ* chemical cross-linking. We show here that stomatin forms major complexes with GLUT1, as anticipated, but also with band 3 and

Abbreviations: ASIC, acid-sensing ion channel; AvTIC, average total ion current; DRM, detergent-resistant membrane; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC-MS/MS, liquid chromatography tandem MS; MS, mass spectrometry; PMSF, phenylmethylsulfonyl fluoride; TNE, Tris/NaCl/EDTA buffer; TNET, Tris/NaCl/EDTA/Triton X-100 buffer

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0005-2736/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbamem.2012.11.030

aquaporin-1. Moreover, we found stomatin to associate with several transporters suggesting a general role as a modulator of transport proteins.

2. Materials and methods

2.1. Reagents

Human blood from healthy donors in EDTA-vials was obtained from the Austrian Red Cross, Vienna. For each experiment, washed red blood cells of 4 donors were pooled. Antibodies were used against stomatin (GARP-50, GARP-61, GARP-65) [1], GLUT1 (Millipore), glycophorin A (Santa Cruz), flotillin 2 (BD Biosciences), and band 3 (Sigma). The cross-linkers ethylene glycolbis(succinimidylsuccinate) (EGS; bridging 16.1 Å distance) and disuccinimidyl suberate (DSS; bridging 11.4 Å distance) were purchased from Pierce/Thermo Scientific; CNBr-activated Sepharose was from Pharmacia/GE Healthcare. Other chemicals of highest purity were from Merck/VWR or Sigma.

2.2. Preparation of erythrocyte membranes

Erythrocytes were purified from 10 ml blood by washing with PBS (3 times 1000 ×*g*), filtration through a column of microcrystalline and α-cellulose [34] and pelleting. Membranes were prepared by hypotonic lysis in 20 volumes of 5 mM EDTA, pH 8.0, 1 mM PMSF (lysis buffer), on ice for 10 min, and centrifugation at 20,000 ×*g* (Sorvall RC5C Plus) for 10 min. The pellet was washed 3 times with lysis buffer. To reduce sample complexity in several experiments, the cytoskeleton was stripped off the membranes by incubating with 10 volumes 0.1 M NaOH on ice for 10 min and washing with lysis buffer.

2.3. Preparation of DRMs

Native membranes were suspended in an equal volume 1% Triton X-100, 5 mM EDTA, 1 mM PMSF in PBS and incubated on ice for 10 min. This mixture was subjected to flotation by mixing with 80% sucrose in PBS (alternatively, in 0.15 M Na_2CO_3 instead of PBS) to yield 50% sucrose, and placed at the bottom of a centrifuge tube. Solutions of 40%, 35% and 5% sucrose in PBS were overlaid sequentially. The samples were centrifuged at 230,000 ×*g* (Beckman Coulter Optima™ L-80 XP ultracentrifuge, SW55Ti rotor) for 16 h. Nine fractions of 0.5 ml were collected from the top and aliquots were analysed by SDS-PAGE/silver staining and Western blotting.

2.4. Chemical cross-linking of membranes

Native or stripped erythrocyte membranes were incubated with 0.8 mM EGS or DSS in PBS, pH 8.0, as recommended by the manufacturer. The reactions were performed on ice for 30 min and stopped by adding 15 mM Tris–HCl, pH 8.0. Respective membrane pellets were solubilised in 1 ml TNET (20 mM Tris–HCl, pH 8.0, 130 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF) at 25 °C for 15 min, cleared by centrifugation (14 000 rpm, Eppendorf, 10 min), and the supernatant was used for immunoisolation of stomatin-complexes. Alternatively, membranes were dissolved in 1% SDS for 5 min at 37 °C, and the solution was diluted with 10 volumes cold (4 °C) TNET before immunoisolation.

2.5. Chemical cross-linking of DRMs

Isolated DRMs were cross-linked with either 16 μM or 8 μM EGS, quenched with 15 mM Tris–HCl, pH 8.0, dissolved in 1% SDS at 37 °C, and the solution was diluted with 10 volumes cold (4 °C) TNET before immunoisolation.

2.6. Immunoisolation of stomatin-complexes

TNET- or SDS/TNET-solubilised, chemically cross-linked membrane proteins were diluted with an equal volume cold (4 °C) TNE (20 mM Tris–HCl, pH 8.0, 130 mM NaCl, 5 mM EDTA, 1 mM PMSF) and loaded onto a small column (1×1 cm) of monoclonal anti-stomatin antibody GARP-50 covalently bound to CNBr-activated Sepharose (1 mg/ml), as described [1]. The column was washed with 15–20 ml 0.1% Triton X-100 in TNE, in some experiments with an intermediate wash with 5 ml 0.3 M NaCl in 0.1% Triton X-100 in TNE, and stomatin complexes were eluted with 5-times 1 ml 0.1 M glycine–HCl, pH 2.5, 0.1% Triton X-100. Each fraction was collected into 55 mM Tris–HCl, pH 8.8, then adjusted to 0.1% SDS, freeze-dried (Speed-Vac), and re-dissolved in 100 μl water.

In summary, twelve independent immunoisolation experiments were performed.

2.7. SDS-PAGE and Western blotting

The immunoaffinity elution fractions were mixed with Laemmli sample buffer, heated for 3 min at 95 °C, and loaded onto 7% or 10% Laemmli SDS-PAGE gels (Hoefer Sturdier SE 400, 14×12 cm) along with HiMark ™ pre-stained high molecular weight standard (Invitrogen). Running conditions for large complexes were up to 24 h at 150 V, 4 °C. In addition, 4–12% gradient gels (GE Healthcare) were used and run for 1 h at 160 V. Gels were silver-stained by a mass spectrometry-compatible method [35] or blotted onto nitrocellulose (16 h at 100 mA, 4 °C) by standard methods. To estimate the relative molecular mass of protein complexes, the Ferguson plot was used. For Western blotting, usually mini-gels (10×8 cm) have been used.

2.8. Mass spectrometry

Silver-stained bands were cut out, proteins digested with trypsin, and the peptides analysed by nano-electrospray LC-MS/MS. Spectra were processed by Mascot 2.2.04 (Matrix Science, London) and the identified peptides were semi-quantitatively estimated by Average Total Ion Current (AvTIC) using the Scaffold3 software (Proteome Software, Portland). Details are given in the Supplementary data. In summary, about 60 MS-analyses were performed.

3. Results

3.1. Isolation and identification of stomatin-complexes after chemical cross-linking of erythrocyte membranes

To generate chemically cross-linked stomatin complexes *in situ*, we incubated normal or cytoskeleton-depleted erythrocyte membranes with 0.8 mM EGS. After membrane solubilisation we isolated the stomatin-complexes by immunoaffinity chromatography and analysed them by SDS-PAGE (Fig. 1A). Due to the massive cross-linking of native membranes, we rather focussed on the cytoskeleton-depleted membranes, because we wanted to target integral membrane proteins, and excised the bands with 130 kDa, 300 kDa, and larger than 500 kDa (Fig. 1A). MS-analysis of these bands clearly revealed the presence of 3 major proteins: stomatin, glucose transporter-1 (GLUT1/SLC2A1), and the anion exchanger (band 3/AE1/SLC4A1) (Fig. 1B). The major component in the >500 kDa band was stomatin, while GLUT1 was highest in the 130 kDa band. In addition to stomatin, GLUT1 and band 3, the >500 kDa band contained flotillin-1 and -2, the urea transporter-1 (UT1/SLC14A1), iron transporter ferroportin-1 (FPN1/SLC40A1), Kell protein/CD238, and protein 4.2, with the urea transporter exceeding GLUT1 and equalling band 3 amounts (Supplementary Fig. 1). While it is known that GLUT1 interacts with stomatin, it is not known for band 3 and the minor proteins. We performed

Please cite this article as: S. Rungaldier, et al., Stomatin interacts with GLUT1/SLC2A1, band 3/SLC4A1, and aquaporin-1 in human erythrocyte membrane domains, Biochim. Biophys. Acta (2012), http://dx.doi.org/10.1016/j.bbamem.2012.11.030