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Reducing isoform complexity of human tetraspanins by optimized expression in *Dictyostelium discoideum* enables high-throughput functional read-out

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ABSTRACT

The human tetraspanin family of scaffold proteins comprises 33 isoforms. Being integral membrane proteins, they organize a so-called tetraspanin web via homomeric and heteromeric protein-protein interactions with integrins, immunoglobulins, growth factors, receptor tyrosine kinases, proteases, signaling proteins, and viral capsid proteins. Tetraspanins promote cellular effects, such as adhesion, migration, invasion, signaling, membrane fusion, protein trafficking, cancer progression, and infections. The ubiquitous expression of multiple tetraspanin isoforms and partner proteins hampers specific interaction studies. Here, we evaluated *Dictyostelium discoideum* as a non-mammalian expression system for human tetraspanins. Using high-content imaging we quantified tetraspanins in *D. discoideum* via fusion with green fluorescent protein. Three human tetraspanins, CD9, CD81, and CD151, served as test cases for which optimizations were carried out. We swapped the GFP domain between the *N*- and *C*-termini, added a Kozak sequence, and partially or fully adapted of the codon usage. This way, CD81 and CD151 were successfully produced. A conformation specific antibody further confirmed correct folding of CD81 and flow cytometry indicated an intracellular localization. Based on these data, we envision a *D. discoideum*-based co-expression platform with human partner proteins for studying tetraspanin interactions and their selective druggability on a large scale without the interference of endogenous human proteins.

1. Introduction

Integral membrane proteins of the tetraspanin family are key components in the organization of membrane-residing proteins into functional complexes, i.e. the tetraspanin web [1,2]. In this web, the tetraspanins form lateral associations with integrins [3,4], immunoglobulins [5–9], growth factors [10–12], receptor tyrosine kinases [13], and proteases [14,15]. For vertical effects, the tetraspanins link signaling proteins to the web, such as protein kinase C [16], phosphatidylinositol 4-kinase [17], and hetero-trimeric G proteins [18–21]. Further, tetraspanins bring about certain direct *trans* interactions in cell-cell-contacts, and with virus capsid proteins [22]. Tetraspanins are typically located at the plasma membrane; however, some isoforms are found intracellularly, such as CD63 that carries a tyrosine-based lysosome targeting motif and is mainly distributed on endocytic organelles [23–25]. On a physiological level, the tetraspanin web is required for cell adhesion, migration, invasion, signaling, membrane fusion, and protein trafficking [25–28]; at the same time, tetraspanins can facilitate cancer progression and infections [28–30].

Despite prominent roles of tetraspanins in (patho-)physiology, in depth biochemical and biophysical examinations of their protein-protein interactions are scarce. This is due to the complexity of 33 isoforms in mammalia [27] resulting in cell systems with multiple tetraspanins present. To address specific questions on tetraspanin interactions, however, a well-defined system is desired in which one can study interactions of interest without the interference of endogenous tetraspanins and additional partner proteins. The only non-mammalian, eukaryotic system used for tetraspanin expression so-far is yeast, yet with the aim to produce high amounts of protein for crystallization [31,32].

Abbreviations: AQP1, aquaporin-1; CAI, codon adaption index; CD, cluster of differentiation; csA, contact site A glycoprotein; DAPI, 4,6-diamidino-2-phenylindole; DdTspanA, *Dictyostelium discoideum* tetraspanin A; DTT, dithiothreitol; GFP, green fluorescent protein; PE, phycoerythrin.

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Further, expression was done in the prokaryotic *Escherichia coli* system and yielded high enough levels to allow subsequent purification [33].

We set out to evaluate another non-mammalian expression system, i.e. the social amoeba *Dictyostelium discoideum*, not for high-yield protein production but for later investigations of human tetraspanin-protein interactions and their putative druggability by small molecules. *D. discoideum* appears to be well suited for the task because only three endogenous tetraspanins are expressed in the amoeboidal stage; at least two of which can be deleted without affecting growth under standard culturing conditions [34]. Further advantages of the system among others concern its genetic accessibility, ease of cultivation, and the eukaryotic nature enabling heterologous expression and post-translational modifications of mammalian membrane proteins, which are often prerequisites for correct folding.

Structurally, tetraspanins are small (20–30 kDa) proteins with four transmembrane spans and short, cytoplasmic *N*- and *C*-termini [35,36]. Palmitoylation at intracellular, juxtamembrane cysteines facilitate interaction with partner proteins [37–41]. Cholesterol and gangliosides stabilize intra-membrane interactions [42–44]. Two extracellular loops connect transmembrane spans 1/2 and 3/4, respectively. The latter, large extracellular loop contains a variable domain and a more conserved, constant region (20% identity among the isoforms) that is thought to be required for homodimerization [45–51]. The variable domain (6.7% identity) harbors a conserved Cys-Cys-Gly (CCG) motif forming the center of a disulphide bridge network within the large extracellular loop yielding a characteristic bifurcated fold [45,47]. This variable domain is proposed to interact with non-tetraspanin protein partners [48].

In this explorative study, we used for expression in *D. discoideum* three human tetraspanin test cases: 1. CD9 (forming, among others, heteromers with tetraspanin CD81 if present) [28]; 2. CD81 (for which most structural data are available) [47,50,52]; and 3. CD151 (the main partner of the integrins) [53]. We established a fluorescence-based high-content imaging method for screening of tetraspanin expression levels by fusion constructs with green fluorescent protein (GFP). We optimized expression by systematic partial and full adaption of the codon usage, and by insertion of a Kozak consensus sequence. Using a conformation specific antibody directed against the large extracellular loop of CD81, we show correct folding of this critical interaction domain. *In vivo* flow cytometry detection of the CD81 accessibility by antibodies revealed an intracellular localization. In conclusion, we found that *D. discoideum* is suited for the expression of human tetraspanins; yet, codon-optimization may be required to maximize expression levels of certain isoforms. Co-expression of interacting partners will be the basis for further studies on the druggability of tetraspanin-protein interactions.

2. Materials and methods

2.1. Generation of the expression constructs

Cloning of human AQP1 was described previously [54]. For cloning into *D. discoideum* expression vectors, the open reading frame was amplified by PCR using the primers GTCAGATCTATGGCAAGCGAGTTCAAG (sense) and GTCACTAGTTTATTTGGGCTTCATCTCCAC (antisense). Amplification of human CD81 and CD151 was done by PCR (One *Taq*, New England Biolabs) with the following primers: CD81 sense ATAAGA TCTATGGGAGTGGAGGGCTGCACCAAGTGCATCAAG and antisense ATAACTAGTT CAGTACACGGAGCTGTTCCGGATGCCACAGCA, as well as CD151 sense ATAAGATC TATGGGTGAGTTCAACGAGAAGAAGACAACATGTGGCACC and antisense ATAACT AGTTTAATAGTGCTCCAGCTTGAGACTCCTGTACAGGCAGCA. Human cDNA from liver and kidney samples was kindly provided by I. Cascorbi. CD9 cDNA was commercially obtained (Bioscience Lifescience, IRATp970C0516D). A contained T644C exchange in CD9 was corrected by PCR with the sense primer ATAAGATCTATGCCGGTCAAAGGAGGCACCA AGTGCATCAAATAC and antisense primer ATAACTAGTCTAGACCATCTCGCGGTTC CTGCGGATAGCACAGCACAAGATCATACTGAA. For the generation of GFP fusion constructs, the open reading frames were ligated into the pDM317 plasmid [55] (*N*-terminal GFP; using *Bgl* II/*Spe* I or, after removal of the stop codon, into the pDM323 plasmid [55] (*C*-terminal GFP). Codon-optimization of CD81 was done using the OptimumGene algorithm (GenScript). The optimized gene with the first 19 codons of contact site A (ATGTC TAGATTTTTAGTATTGATAATATTATATAATATTTTAAATAGTGCACATTCA) upstream of the start codon, connected by a linker including a Kozak sequence (GTCGACAA AGATCTAAAA), was synthesized (GenScript). Adaptation of the first ten codons of CD151 and insertion of the Kozak sequence was done by insertion of a synthesized linker (sense GATCTGACGTCAAAAATGGGTGAATTTAATGAAAAAAAAAACTACTTGTGG CACCGTTTGCCTCAAGTACCTGCTGTTTACCTAC, antisense AATTGTAGGTAAACA GCAGGTACTTGAGGCAAACGGTGCCACAAGTAGTTTTTTTTTCATTAAATTCACCCATTTTTGACGTCA) using *Bgl* II and *Mfe* I. All constructs were verified by DNA sequencing.

2.2. D. discoideum cell culture, transformation and Western blot

AX2 cells were cultured axenically at 22 °C in HL5 medium (Formedium) with 0.5% glucose [56]. For expression of the GFP fusion constructs, *D. discoideum* amoeba from 24 h shaking cultures were harvested by centrifugation (2000 *g*, 10 min, 4 °C) and washed twice with H50 Buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$ and 1 mM NaH_2PO_4, pH 7.0). 7×10^6 cells were suspended in 700 µl H50 buffer and incubated for 5 min with 6–10 µg of plasmid DNA. Electroporation was done in 0.4 mm cuvettes with a Gene Pulser II (BioRad) using two pulses with 5 s delay of 1.2 kV, 50 µF, time constant 1–2 ms. The cells were chilled on ice, supplemented with HL5 medium containing 0.5% glucose, and incubated for 16–24 h at 22 °C. Transformed cells were selected by addition of 10 µg ml^{-1} G418 (Calbiochem). For Western blot analysis, *D. discoideum* amoeba in shaking cultures were harvested in mid-log phase (2000 *g*, 10 min, 4 °C), resuspended in 1 ml water, and lysed by repeated freeze (−80 °C) and thaw (37 °C) cycles. The lysates were solubilized for 30 min at 37 °C in SDS loading buffer (250 mM TRIS, pH 6.8, plus 8% SDS, 40% glycerol, 400 mM dithiothretiol, 0.02% bromophenol blue). 30–170 µg of total protein per lane were separated by SDS-PAGE in 12.5% gels and electro-transferred to PVDF membranes (Amersham). Polyclonal rabbit anti-GFP, polyclonal anti-hAQP1 antisera, and a monoclonal mouse anti-CD81 antibody (sc-23962) were from Santa Cruz Biotechnology. Secondary anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase (Jackson Immuno Research Laboratories) were used for detection with ECL plus reagents (GE Healthcare).

2.3. Osmoregulation assay

The assay was done as described previously [34]. Briefly, using a high-content imaging system (ImageXpressMicro, Molecular Devices) with a 100 × objective, cells in HL5 medium with 0.5% glucose were initially imaged (t = 0 min), then the medium was exchanged with distilled water, and the cells were monitored for 20 min. Cellular circularity was determined offline for individual