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A generic high-throughput assay to detect aquaporin functional mutants: Potential application to discovery of aquaporin inhibitors

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ABSTRACT

Background: The discovery of stable, yet functional, protein mutants is a limiting factor in the development of biotechnological applications, structural studies or in drug discovery. Rapid detection of functional mutants is especially challenging for water channel aquaporins, as they do not have a directly measurable enzymatic or binding activity. Current methods available are time consuming and only applicable to specific aquaporins.
Methods: Herein we describe an assay based on the protective effect of aquaporins on yeast *S. cerevisiae* in response to rapid freezing.
Results: Yeast overexpressing a functional water-permeable aquaporin of choice are rescued after the challenge, while inactive or blocked aquaporins confer no protection and lead to cell death. The potential of this assay is shown by screening a small number of *E. coli* aquaporin Z (AQPZ) mutants. Additionally, a library of ~10,000 drug-like compounds was tested against human AQP1 (hAQP1).
Conclusions: Since rescue is only dependent on transmembrane water flux, the assay is applicable to water-permeable aquaporins of any origin.
General significance: Mapping of permissive mutations on the aquaporin structure can help delineate the minimal requirements for effective water transport. Alternatively, the assay can be potentially used to discover compounds that inhibit aquaporin water transport. When additionally screened for thermostability, functional aquaporin mutants can be useful in the development of biomimetic membranes for water purification, or to improve the likelihood of producing well-diffracting crystals, enabling rational design of much needed aquaporin inhibitors.

1. Introduction

Aquaporins (AQPs) are integral membrane proteins that transport water through cellular membranes [1–3]. AQPs are found in all organisms, from bacteria to humans [4–10], although with different distribution. In humans there are 13 different AQPs [11], with important physiological roles (reviewed in [12]), of which five are water-specific (orthodox AQPs): AQP0, 1, 2, 4, 5 and 8 [11,13]. The architecture of AQPs is conserved from bacteria to humans [14,15]: the functional form is a homo-tetramer, where each AQP monomer has six transmembrane (TM) α-helical domains and functions independently as a water channel. Each AQP monomer can transport as many as 3 billion water molecules per second, while rejecting all other solutes, including protons [16]. Such high permeabilities and selectivities depend on geometric and physico-chemical factors that are now beginning to be understood, as recently shown for a yeast aquaporin [17]. In this respect, the availability of aquaporin mutants bearing several mutations, while still preserving function, would be helpful in understanding water permeability and selectivity. This is particularly true when data is obtained from several aquaporin homologs, and when discovered mutations are located at sensitive pore-lining surfaces. Also, these functional mutants may have higher thermal stability, which is a desirable property in biotechnological applications. Since our assay is dependent of water permeability through aquaporin, the protocol we describe herein can also be adapted as a generic screen to detect AQP water channel inhibitors.

In humans, AQP inhibition or regulation has tremendous potential for medical applications [18,19]. For example, water channel inhibitors, e.g., against hAQP1, could be used to discern the role of enhanced water flow caused by hAQP1 overexpression during cell migration and angiogenesis [20,21]. Most current AQP inhibitors are toxic or unsuitable leads for drug discovery [22–24], and other compounds lack sufficient affinity and specificity when tested in erythrocyte-based assays [25–27].

Current assays to screen for modulators of AQP water transport monitor biophysical parameters related to cell volume change, e.g., light scattering or fluorescence changes due to rapid cell shrinkage caused by osmotic shock [28,29], but these assays are not generic and therefore

Abbreviations: AQPs, aquaporins; *S. cerevisiae*, *Saccharomyces cerevisiae*; YPD, yeast extract + peptone + dextrose; NCI, National Cancer Institute; DMSO, dimethyl sulfoxide; OG, n-octyl-β-D-glucopyranoside; RBC, red blood cell

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unsuitable as a general screen for inhibitors against all the different subtypes of AQPs.

The protocol we describe is based on the previously observed correlation between the degree of tolerance of yeast to freeze–thaw and the expression levels of yeast aquaporin genes AQY1 and AQY2 [30,31]. Indeed, deletion of AQY genes increased the yeast sensitivity to freezing, whereas overexpression of AQPs (AQY1/AQY2 or hAQP1) improved freeze tolerance. This protection during rapid freezing is thought to be conferred by AQPs as they facilitate water efflux through the plasma membrane, thereby preventing formation of intracellular cell-damaging ice crystals [30,31].

In agreement with the latter, we have found that *S. cerevisiae* lacking native AQPs, but overexpressing a functional water-permeable human or bacterial AQP of choice, are rescued from a freezing challenge. In contrast, yeast over-expressing mutants known to be inactive, or yeast expressing wild type aquaporins but exposed to known aquaporin inhibitors, cannot be rescued. The proof of principle of this assay is shown by screening a small library of 160 AQPZ random mutants, where only a handful of colonies survived to the freeze–thawing treatment. The corresponding AQPZ mutations in these colonies localized to residues away from the water pore. We also show the results of the application of this assay in the screening a small library of ~10,000 drug-like compounds against *S. cerevisiae* expressing hAQP1.

2. Materials and methods

2.1. Generation of a random mutagenesis library of E. coli aquaporin Z (AQPZ)

Mutagenic, error-prone PCR was performed on 90 ng of the AQPZ cDNA using GeneMorph® II Random Mutagenesis Kit (Stratagene, CA). 2.5 U of Mutazyme II DNA polymerase was used per reaction. Acting as megaprimers, the resultant fragments were annealed to template plasmid YEp181-PGK-His_6-AQPZ. Whole plasmid PCR amplification was carried out using 2.5 U of *Pfu* DNA polymerase (Promega, USA). Products treated with 20 U of *Dpn*I (New England Biolabs, UK) and incubated at 37 °C for 3 h were transformed into yeast.

2.2. Yeast transformation

To generate *S. cerevisiae* cells stably expressing AQP, YEplac181 vector containing a PGK promoter sequence upstream of the AQP cDNA sequence was used. The AQP-null wild-type laboratory *S. cerevisiae* strain BY4743 (MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0) (EUROSCARF, Germany) was transformed using *S. c.* EasyComp Transformation Kit (Invitrogen, USA) according to the manufacturer's instructions. Transformants screened on selective agar containing 1.6 g/L yeast synthetic drop-out medium supplements without leucine (SD-Leu) (Sigma) were incubated at 30 °C for 2 to 3 days. For cell imaging, an enhanced green fluorescence protein sequence was added downstream of the AQP cDNA sequence. Mutants identified by the freeze–thaw assay (see below) were picked and inoculated into 4 mL of SD-Leu medium and cultured overnight at 30 °C. Cells were harvested by centrifugation at 13,000 rpm for 1 min, followed by lysis with glass beads. Plasmids were extracted and sequenced, and mutations were mapped onto the AQPZ structure (Protein Data Bank accession code: 1RC2, [32]) using Pymol (Delano Scientific).

2.3. Western blotting

To detect protein expression, samples were prepared as described previously [33], with modifications. Briefly, transformed yeast was pre-cultured overnight in 10 mL SD-Leu. 50 mL of YPD was inoculated to $OD_{600} = 1$. After culturing for 6 h, cells were harvested and resuspended in cell resuspension buffer (CRB) consisting of 20 mM Tris–HCl pH 7.6, 100 mM NaCl, 1 mM EDTA and 5% glycerol, and glass beads were added (cellpellet:CRB:glassbeads ratio of 1:1:1). Cells were broken by 6 vortex-ice cycles (each cycle = 30 s intensive vortexing and 30 sec ice cooling). Samples were centrifuged at 500 *g* for 10 min at 4 °C to obtain the crude extract (500 *g* supernatant), which was then centrifuged at 10,000 *g* for 30 min followed by 100,000 *g* for 90 min at 4 °C to obtain the membrane pellet (membrane fraction). The crude extract and membrane fraction were diluted in sample buffer (50 mM Tris–HCl pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol Blue), denatured at 65 °C for 10 min and separated by SDS-PAGE, followed by transfer to a PVDF membrane and analysis by Western blotting (mouse anti-His, Qiagen 34660 or mouse anti-hAQP1, Abcam ab117970). For AQPZ, detection was not possible when the His-tag was at the N-terminus, possibly because enzymatic cleavage or inaccessibility. Therefore, the His-tag was changed to the C-terminus, and in this case AQPZ could be detected. A control freeze–thawing experiment showed that AQPZ with a C-terminal His-tag is equally able to rescue yeast after freeze–thawing (not shown).

2.4. Freeze–thaw assay

Yeast colonies were inoculated into flat-bottomed 96-well microplates containing 200 μL of YPD [1% (*w*/*v*) yeast extract, 2% (*w*/*v*) bactopeptone with 2% dextrose] medium per well. The microplates were incubated overnight at 30 °C with orbital shaking. Overnight cultures were adjusted to a cell density of 6×10^6 cells/mL. Plates were sealed and incubated for 15 min. The control plate (96-well flat-bottom plate, Greiner, UK) was stored at 4 °C while the treatment plate (96-well thin-walled PCR Plate, SSI, CA) was rapid frozen by plate submersion into liquid N_2 for 30 s, and thawed in a room temperature water bath for 20 min. This freeze–thawing procedure was applied twice.

2.5. Viability measurement

Yeast cell viability was measured using a microbial viability WST assay kit (Dojindo, Japan) according to the manufacturer's instructions. Briefly, 10 μL of the coloring reagent was added to 190 μL of cell culture in each well of a 96-well microplate. After 6 h at 30 °C, absorbance at 450 nm was measured using a microplate reader (Tecan M200 Pro, Austria), with the reference measurement at 600 nm. Alternatively, after overnight incubation at 30 °C, the OD_{600} was measured. Percentage cell viability in plates containing the AQPZ mutants was defined as [(OD_{600} treatment / OD_{600} control) × 100]. Mutants with viability of >50% were selected and rescreened. The quality of the screening assay was determined by a Z′ factor, calculated according to Eq. (1) [34],

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} + \mu_{c-}|} \quad (1)$$

where μ_{c+} and μ_{c-} are the average values of yeast expressing AQP (positive controls) and yeast with vector alone (negative controls) after freeze–thawing, and σ_{c+} and σ_{c-} are the respective standard deviations. Both μ_{c+} and μ_{c-} were calculated as the average of 96 wells.

2.6. Compound library screening

The drug library screened was a collection of ~10,000 compounds (National Cancer Institute, NCI, USA), including the approved Oncology Drugs Set, Diversity Set, Mechanistic Diversity Set, Natural Product Set and Open Set. BY4743 cells were cultured overnight at 30 °C in YPD medium and adjusted to a cell density of 6×10^6 cells/mL. In a control plate (96-well flat-bottom plate), each compound was tested at 10 μM on yeast expressing hAQP1 in a total volume of 200 μL (final DMSO 1%). A volume of 100 μL from this control plate was transferred to a treatment plate (96-well thin-walled PCR Plate). Both plates were sealed and incubated for 15 min. The control plate was stored at 4 °C, while the treatment plate was freeze–thawed as described. Compounds that