Analytical Biochemistry 549 (2018) 58–65

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Spherical-supported membranes as platforms for screening against membrane protein targets

V. Vasilca[a], A. Sadeghpour[b,c], S. Rawson[a], L.E. Hawke[a], S.A. Baldwin[a,1], T. Wilkinson[d], D. Bannister[d], V.L.G. Postis[a,e], M. Rappolt[b], S.P. Muench[a], L.J.C. Jeuken[a,*]

[a] The School of Biomedical Sciences and the Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, LS2 9JT, United Kingdom
[b] School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, United Kingdom
[c] Center for X-ray Analytics, Department of Materials Meet Life, EMPA, 9014 St Gallen, Switzerland
[d] MedImmune, Antibody Discovery and Protein Engineering, Milstein Building, Granta Park, Cambridge, CB21 6GH, United Kingdom
[e] Biomedicine Research Group, Faculty of Health and Social Sciences, Leeds Beckett University, Leeds, United Kingdom

A B S T R A C T

Screening assays performed against membrane protein targets (e.g. phage display) are hampered by issues arising from protein expression and purification, protein stability in detergent solutions and epitope concealment by detergent micelles. Here, we have studied a fast and simple method to improve screening against membrane proteins: spherical-supported bilayer lipid membranes ("SSBLM"). SSBLMs can be quickly isolated via low-speed centrifugation and redispersed in liquid solutions while presenting the target protein in a native-like lipid environment. To provide proof-of-concept, SSBLMs embedding the polytopic bacterial nucleoside transporter NupC were assembled on 100- and 200 nm silica particles. To test specific binding of antibodies, NupC was tagged with a poly-histidine epitope in one of its central loops between two transmembrane helices. Fluorescent labelling, small angle X-ray scattering (SAXS) and cryo-electron microscopy (cryo-EM) were used to monitor formation of the SSBLMs. Specific binding of an anti-his antibody and a gold-nitrilotriacetic acid (NTA) conjugate probe was confirmed with ELISAs and cryo-EM. SSBLMs for screening could be made with purified and lipid reconstituted NupC, as well as crude bacterial membrane extracts. We conclude that SSBLMs are a promising new means of presenting membrane protein targets for (biomimetic) antibody screening in a native-like lipid environment.

Introduction

Encoded by almost one third of archaean, bacterial and eukaryote DNA [1], membrane proteins represent vital cellular components for all life-forms. Given their essential roles towards sustaining life, it is unsurprising that membrane protein pathology accounts for a large number of debilitating conditions, such as Bartter syndrome, cardiac arrhythmia and hypertension, congenital deafness and myotonia, cystic fibrosis, epilepsy, osteoporosis and polycystic kidney disease [2,3]. Their significant therapeutic importance has led to many of today's pharmaceuticals targeting membrane proteins [4,5], with the largest class being the G-protein coupled receptors (GPCRs). However, the discovery of novel membrane protein binders – including antibody-based medicines that have emerged throughout the last decade [6] – is not without issue. The high-throughput protocols employed by the drug discovery industry demand high levels of expression and purity from their designated screening targets, yet few membrane proteins can be expressed at high level within their native membranes. Moreover, the general study of membrane proteins is further complicated by the fact that advanced research techniques (e.g., kinetic and ligand-binding characterisation, nuclear magnetic resonance (NMR) or X-ray crystallography) cannot always be directly performed on crude cellular membranes and thus require generous amounts of recombinant protein of high purity and conformational stability, therefore becoming reliant on identifying optimised expression platforms, a suitable detergent for the solubilisation and, more often than not, demanding high-throughput methodologies [7–9].

Unfortunately, systems used in the overproduction of membrane protein targets rarely express high amounts of recombinant protein [10], partly due to differences between the biogenesis pathways of the host and those of the expression systems and/or the imposed xenobiotic toxicity [8]. Even following successful expression, membrane proteins are notoriously difficult to purify via standard techniques such as ion exchange or hydrophobic interaction and poor overall yields can still be registered after the inclusion of specialised high-affinity

* Corresponding author.
E-mail address: L.J.C.Jeuken@leeds.ac.uk (L.J.C. Jeuken).
[1] Deceased author.

https://doi.org/10.1016/j.ab.2018.03.006
Received 25 January 2018; Received in revised form 9 March 2018; Accepted 12 March 2018
Available online 13 March 2018
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chromatography tags [11]. Furthermore, target denaturation is an ever-present concern after the proteins have been removed from their native membranes and this is the main reason why detergent solubilisation has been traditionally used to counter the considerable hydrophobicity and aggregation tendency of membrane proteins post-purification [7,9]. While detergent-solubilised proteins facilitate screening with other biomolecules such as ligands or inhibitors in solution [12], it is commonly desirable to transfer the target proteins into less disruptive environments, since even the mildest detergents can still lead to the complete inactivation of the solubilised proteins [7]. Moreover, in the context of antibody binding studies, detergent micelles can also actively block potential epitopes on the chosen screening targets and can thus have a direct negative impact on the discovery of new antibody-based pharmaceuticals [12,13].

The main objective of the research presented here was therefore to develop an alternative screening platform based on spherical-supported bilayer lipid membranes ("SSBLMs"), which can present membrane protein targets in a native-like lipid environment. SSBLM consist of a solid spherical core, typically silica, which is coated with lipid membranes. SSBLMs were first developed in the 80s and 90s, are well characterised with spectroscopy and microscopy and their formation has been well documented (see Ref. [14] for a review on SSBLMs). SSBLMs have already been reported for several membrane proteins, such as the multidrug efflux pump component OprM [15], bacteriorhodopsin [16] or the redox-driven proton pump cytochrome *c* oxidase [17]. This prompted us to explore whether, by refinement of the SSBLM format, this technology can be used in assays that require or select for specific, high-affinity antibody binding and, eventually, screening assays. In order to enhance the amount of protein presented in a screening assay, submicron silica particles were used.

In order to provide proof-of-concept for our proposed screening platform, the bacterial nucleoside transporter NupC was chosen as the membrane protein of interest. Involved in active (secondary) transport of both purine and pyrimidine nucleosides across bacterial inner membranes (IMs), NupC is a proton-dependent symporter belonging to the concentrative nucleoside transporter (CNT) family [18–20]. The protein shares 22–26% amino acid sequence identity with the human transporters hCNT1-3, which renders it a good model for studying the transport of the therapeutic nucleoside analogues used in the treatment of life-threatening viral and neoplastic diseases, such as azidothymidine and gemcitabine [21]. Since antibody-based pharmaceuticals are typically expected to target epitopes located in the loop regions of transmembrane proteins, a clone of NupC was engineered to feature a His-tag on one of its central loops, between two transmembrane helices. This affinity tag allowed for the binding of both anti-His antibodies as well as gold-conjugated nitrilotriacetic acid (NTA) probes, which greatly aided us in providing our proof-of-concept.

Here, we show that SSBLMs are a suitable platform for screening assays and report on technical improvements that are required to reduce non-specific binding of antibodies to the SSBLM particles. Non-specific binding of proteins, including antibodies and biomimetic antibodies, can occur if silica particles are not completely coated by the lipid membranes, exposing some of the bare silica surface [22]. Here, we show that including liposomes and bovine serum albumin (BSA), but not detergents, during the incubation steps with antibodies is a simple and effective strategy to block non-specific binding. Furthermore, we show that this method can also be applied when using crude membrane extracts, negating the need to tag and purify membrane proteins in screening assays.

Materials and methods

Materials

All chemicals were purchased from Sigma-Aldrich or Melford unless stated otherwise. His-tagged NupC detection employed HRP-conjugated mouse IgG_1 anti-His antibodies (R&D Systems, MAB050H). Lipid, detergent and related materials included 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids (Avanti Polar Lipids, 850457), *α*-[4-(1,1,3,3-Tetramethylbutyl)phenyl]-*w*-hydroxy-poly(oxy-1,2-ethanediyl) (Triton X-100) (10% (w/v) solution) (Anatrace, APX100), Bio-Beads SM-2 adsorbent beads (Bio-Rad, 1523920) and Texas Red 1,2-Dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (TR-DHPE) (Thermo Fisher Scientific, T1395MP). Silicon dioxide (SiO_2) spheres with diameters of 100- and 200 nm were supplied as 10 mg/mL aqueous solutions (nanoComposix, SISN100 and SISN200, respectively). The peroxidase assay employed a SensoLyte 10-Acetyl-3,7-dihydroxyphenoxazine (ADHP) peroxidase assay kit (fluorimetric) (AnaSpec, AS-71111). Cryo-EM materials included 5 nm Ni-NTA-Nanogold probes (Nanoprobes, 2082) and lacey carbon film/copper mesh cryo-grids (Agar Scientific, AGS166).

NupC cloning

Both an untagged version (pGJL16) as well as a His-tagged construct, of NupC (pLH13), were used. The plasmid pGJL16 was obtained by cloning the *E. coli nupC* gene into a pTTQ18 vector [23] between *Eco*RI and *Hin*dIII. pTTQ18 features an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible *tac* promotor [23]. pLH13 was then cloned from pGJL16 through the insertion of a pentahistidine tag. We previously reported that cloning a His-tag into either the N- or C-terminus of NupC prevents its expression [24], hence a pentahistine tag was inserted in the central cytoplasmic loop between transmembrane (TM) helices 5 and 6, specifically between His230 and Glu231. The pentahistine tag, along with the native His230, thus resulted in 6 consecutive histidines. In pLH13, Cys96 was also mutated to an Ala to reduce potential dimerisation and aggregation. While the uridine uptake activity of the internally His-tagged NupC construct was substantially reduced compared to the wild-type variant, its post-purification functionality was nevertheless retained (unpublished results).

Purification of His-tagged NupC

The purification of the His-tagged NupC was modified from Ref. [24]. pLH13 was transformed into *E. coli* strain C43 and grown in Lysogeny broth (LB) media supplemented with 100 μg/mL carbenicillin. C43/pLH13 was cultured as 500 mL cultures in 2 L baffled flasks at 37 °C with 200 rpm orbital shaking until reaching an OD_{600nm} of ~0.6, after which expression was induced with 1 mM IPTG (Generon) for another 4 h. The cells were then harvested via centrifugation (9000 × *g* for 20 min) and resuspended in 20 mM Tris, 0.5 mM EDTA (pH 7.4) using volumes five to six times the weight of the harvested cells. Once resuspended, the cells were homogenised using an Ultra-Turrax cell homogeniser and subsequently lysed via two consecutive runs through a TS5/40/AB/GA cell disrupter (Constant Systems) at 30 kPsi. The lysed cells were centrifuged at 14,000 × *g* for 45 min in order to remove cellular debris. The supernatant was ultracentrifuged at 131,000 × *g* for 2 h to isolate the bacterial membranes. The protein concentration of the membrane preparation was determined using the bicinchoninic acid (BCA) assay. The membranes were solubilised in solubilisation buffer (1% (w/v) n-Dodecyl-β-D-maltoside (DDM), 50 mM phosphate buffer, 10% (w/v) glycerol, 150 mM NaCl, 5 mM imidazole and cOmplete™ (EDTA-free) mini protease inhibitor cocktail, pH 7.4) at 4 °C for 1 h at a total membrane protein concentration of approximately 5 mg/mL. The solubilised membranes were then ultracentrifuged at 110,000 × *g* for 1 h, after which the insoluble pellet was discarded. The supernatant was added to a bed volume of 80 μL of cobalt affinity chromatography resin (Pierce) per 25 mg of total membrane protein, pre-equilibrated in wash buffer (50 mM phosphate buffer, 10% (w/v) glycerol, 150 mM NaCl, 5 mM imidazole and 0.05% (w/v) DDM, pH 7.4). NupC was bound to the resin for 16 h at 4 °C with gentle roller mixing. The resin was packed into a disposable filtered column (Thermo-Pierce Scientific) and

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