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Surveying GPCR solubilisation conditions using surface plasmon resonance

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ABSTRACT

Biophysical screening techniques, such as surface plasmon resonance, enable detailed kinetic analysis of ligands binding to solubilised G-protein coupled receptors. The activity of a receptor solubilised out of the membrane is crucially dependent on the environment in which it is suspended. Finding the right conditions is challenging due to the number of variables to investigate in order to determine the optimum solubilisation buffer for any given receptor. In this study we used surface plasmon resonance technology to screen a variety of solubilisation conditions including buffers and detergents for two model receptors: CXCR4 and CCR5. We tested 950 different combinations of solubilisation conditions for both receptors. The activity of both receptors was monitored by using conformation dependent monoclonal antibodies and the binding of small molecule ligands. Despite both receptors belonging to the chemokine receptor family they show some differences in their preference for solubilisation conditions that provide the highest level of binding for both the conformation dependent antibodies and small molecules. The study described here is focused not only on finding the best solubilisation conditions for each receptor, but also on factors that determine the sensitivity of the assay for each receptor. We also suggest how these data about different buffers and detergents can be used as a guide for selecting solubilisation conditions for other membrane proteins.

Introduction

G-protein coupled receptors (GPCRs) are the largest class of drug targets due to the size of the gene family in the human genome and the breadth of extracellular ligands members that GPCRs have evolved to respond to. GPCRs are integral membrane proteins containing seven transmembrane α-helices. The membrane-bound nature of GPCRs is a persistent challenge to the application of modern biophysical techniques routinely used in drug discovery. In recent years there have been advances in exploiting biophysical methods, such as X-ray protein crystallography and surface plasmon resonance, for screening GPCRs [1–3]. However, in order to carry out biophysical studies, recombinant GPCRs must first be isolated from the membrane with the hydrophobic transmembrane regions embedded in an appropriate environment. In order to facilitate their analysis two general strategies have been employed to stabilise them outside of their natural membrane environment. One set of strategies employs conformational stabilisation of the GPCRs by either mutagenesis to increase the thermostability of the protein [2–4] or by the formation of stable complexes [5] (e.g. with antibody fragments or nanobodies). These methods have proven successful for both crystallizing GPCRs for X-ray analysis and also for surface plasmon resonance (SPR). A second strategy, generally but not exclusively used in conjunction with the first strategy, is to stabilise the transmembrane region of the receptor outside of its native environment by using a membrane mimetic system. Membrane mimetic systems generally involve solubilisation of the receptor in detergents or in an artificial membrane [6–8]. The advantage of solubilisation techniques is that, unlike stabilisation through mutagenesis, where stability arises from locking the structures in one conformation, the full range of tertiary structure conformations and thus full pharmacological function remains. Solubilisation methods have proved particularly useful in enabling SPR to be applied to the study of fully functional, 'wild type' GPCRs for screening of antibodies, small molecule drugs and low molecular weight 'fragment' compounds (Mw < 250 Da) [9–11]. Despite the potential for solubilisation strategies to expand the repertoire of GPCRs that can be studied by SPR screening, there has been relatively little systematic analysis of the landscape of detergent, lipid and buffer mixtures that result in active wildtype receptors being immobilised on a biosensor surface.

To better understand the range of solubilisation conditions that are tolerated by active GPCRs, we undertook a large-scale screen of buffer and detergent solubilisation conditions using surface plasmon

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resonance based biosensors on two model receptors: CXCR4 and CCR5. Smaller scale detergent screens using SPR have been described previously [11,12]. We have expanded upon previous work by extending the search space by testing 950 different combinations of solubilisation conditions for two receptors. Conformation dependent monoclonal antibodies specific to active properly folded proteins were used to monitor the functional activity of CXCR4 and CCR5. Antibodies are useful as a tool for biosensor assay because their high molecular weight provides a larger response signal in SPR and therefore increases the sensitivity of the method.

Materials and methods

The Biacore 3000, Biacore 4000, and Biacore T200 biosensors, CM4 sensor chips, CM4 Series S sensor chips, and the amine coupling kit were supplied from Biacore AB (Uppsala, Sweden). The MASS-1 biosensor, high capacity amine sensor chips and amine coupling kit were obtained from Sierra Sensors (Hamburg, Germany). The 1D4 antibody was purchased from the University of British Columbia. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Cf2Th-CXCR4 from Dr. Joseph Sodroski [13]. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Cf2Th synCCR5 + Cells from Dr. Tajib Mirzabekov and Dr. Joseph Sodroski [14]. Both receptors were expressed with a C-terminal linear C9 peptide tag (TETSQVAPA) specific for the 1D4 antibody. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Maraviroc (Cat #11580). Lipids (synthetic phospholipid blend DOPC:DOPS [7:3, w/w] were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Detergents n-dodecyl-b-D-maltoside, n-octyl-b-D-glucopyranoside, Chaps, n-tridecyl-b-D-maltopyranoside, Big Chap deoxy, DSOL-MK – Solution Master Detergent Kit, were purchased from Anatrace (Maumee, OH, USA). Cholesteryl hemisuccinate tris salt was purchased from Sigma-Aldrich. Complete, EDTA-free, protease inhibitor tablets were purchased from Roche Diagnostics. The It1t compound was purchased from Tocris. The 2D7 antibody was purchased from BD Biosciences. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: CXCR4 Monoclonal Antibody (12G5) from Dr. James Hoxie [15]. Additional 12G5 antibody was purchased from R&D systems.

CCR5 and CXCR4 expression

Cf2Th/syn CCR5 and Cf2Th-CXCR4 cells were thawed in a water bath at 37 °C. The thawed cells were then transferred to a 50 ml falcon tube and 10 ml of medium was added. The medium was composed of 90% Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS) supplemented with pen/strep. The cells were spun for 5 min at 3000 rpm at room temperature. The cell pellet was suspended in 5 ml of fresh medium and the sample was diluted 1:40 for CCR5 and 1:160 for CXCR4 in T25 flasks containing 8 ml of medium and incubated at 37 °C until confluent. The cells were then split following release from the flask surface by trypsin/EDTA treatment. Prior to trypsinolysis the cells were briefly washed with PBS. Once fully recovered, the cells were grown in adherent cell culture and DMEM medium supplemented with the antibiotics: 500 μg/ml G418, 500 μg/ml zeocin, 3 μg/ml puromycin for CCR5, and 0.4 mg/ml G418 for CXCR4. The cells were harvested, pelleted in 2×10^6 and 0.4×10^6 cell aliquots and frozen at −80 °C until required.

Preparation of lipids

Lipids were prepared using a similar procedure to that described previously [9,11]. Lipid mixture DOPC:DOPS [7:3, w/w] was diluted in chloroform and transferred to glass vials in a volume resulting in 5 μMoles in the vial. The chloroform was evaporated using nitrogen gas thus forming a thin layer of lipid on the glass vial. To remove any residual chloroform the samples were placed under vacuum for 6–8 h. The dried lipids were stored at −80 °C until required. For preparation of a working solution, the thin lipid layer was solubilised in 1 ml of buffer comprised of 50 mM HEPES (pH 7.0), 150 mM NaCl to give a final concentration of 5 mM. Four cycles of vortex, freeze, and thaw were performed. The lipids were then stored at 4 °C.

1D4 antibody immobilisation

For the Biacore 3000, the monoclonal antibody 1D4 was immobilised on a CM4 sensor chip using standard amine-coupling chemistry in an assay buffer composed of 10 mM HEPES (pH 7.4), 150 mM NaCl at 25 °C. The sensor chip surface was activated using 400 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 100 mM N-hydroxy succinimide (NHS) mixed 1:1. The EDC/NHS solution was injected for 10 min at a flow rate of 10 μL/min. The 1D4 antibody, diluted in 10 mM sodium acetate (pH 5.0), was then coupled to the surface using a 15-min injection at flow rate of 5 μL/min followed by a 7-min injection of 1 M ethanolamine (pH 8.5) at flow rate of 10 μL/min to block any remaining activated groups on the surface. The antibody density achieved was 8–12000 RU. For antibody immobilisation on the Biacore 4000, T200 and MASS-1 the temperature was 30 °C.

Preparation of solubilisation buffers

The buffers were prepared from 2 M stock solutions of HEPES (pH 7.0), TRIS (pH 7.0), NaCl and $(NH_4)_2SO_4$, 50% glycerol, 50% PEGs 400, 1500, 4000, 6000 and 8000, sucrose and additional salts diluted in purified water to desired concentrations. The pH of the final buffers was kept at 7.0. The exact composition of solubilisation buffers is summarised in Supplementary Table 1.

CCR5 and CXCR4 solubilisation buffer screen

Cf2Th/syn CCR5 cells (0.4×10^6) or Cf2Th-CXCR4 cells (2.0×10^6) were suspended in 180 μL of solubilisation buffer containing protease inhibitors. For CCR5, the sample was supplemented with 0.5% DDM, 0.5% CHAPS, 0.1% CHS and 0.25 mM DOPC/DOPS (7:3, w/w) and for CXCR4, the sample was supplemented with 0.33% DDM, 0.33% CHAPS, 0.07% CHS and 0.33 mM DOPC/DOPS (7:3, w/w) to final volume of 200 μL. The cell suspensions were briefly sonicated and then incubated on a rocker at 4 °C. After 6–8 h incubation, the samples were spun at 14,000 rpm for 20 min at 4 °C. The supernatant was carefully transferred to a new vial and used for capture over the immobilised 1D4 antibody surface on the Biacore 4000 or Biacore 3000 for the CCR5 or CXCR4 receptors respectively. For the Biacore 4000 assays the receptor, solubilised under specific conditions, was captured over the antibody surface. Four solubilised receptors can be captured in a single injection step and due to the number of spots in each flow cell, samples were captured on both spot 1 and 5 of each flow cell, leading to the total of 8 solubilisation conditions monitored per cycle e.g. four conditions were captured over spot 1 and four over spot 5, using spot 3 as a reference for each flow cell. For the Biacore 3000 the solubilised receptor was captured on three flow cells leaving one flow-cell empty as a reference.

The solubilised receptor was injected for 240 s at flow rate of 5 μL/min. Running buffer was injected for 180 s to stabilise and wash the surface, followed by injection of 2D7 (CCR5) or 12G5 (CXCR4) antibody for 60–180 s (at concentrations of 40 or 50 nM respectively). Dissociation was monitored for 120 s. The flow rate was set to 30 μL/min. The surface was regenerated with 2 × 10 s-pulses of 10 mM NaOH/1% n-octyl-β-D-glucopyranoside solution at flow rate of 30 or 50 μL/min followed by a wash with running buffer for 60 s. Control samples are receptors solubilised in the previously established solubilisation conditions [11] (20 mM TRIS hydrochloride pH 7.0, 100 mM Ammonium sulphate, 10% glycerol and for CXCR4 only 5 mM $MgCl_2$