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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 molecules 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.

Keywords: GPCRs, Surface plasmon resonance, Solubilisation conditions, Detergents, Screening

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 stabilize 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 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 the 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 minutes 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]. Briefly, 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 hours. 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 ºC. 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 minutes 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-minute injection at flow rate of 5 µl/min followed by a 7-minute 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 ºC.

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 hours incubation, the samples were spun at 14,000 rpm for 20 minutes 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 x 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 sulfate, 10% glycerol and for CXCR4 only 5 mM $MgCl_2$ and 1 mM $CaCl_2$) and were included in the screen for reference and comparison purposes. The control samples were injected at the beginning and end of the screen for the CCR5 receptor. Due to the larger number of samples tested per day on the Biacore 3000 the control sample was injected throughout, including the beginning and end of the CXCR4 receptor screen. In order to avoid a potential risk of blocking the instrument microfluidics the solubilisation conditions were screened in increasing molecular weight of PEGs due to the high viscosity of the buffers.

The running buffer for CCR5 receptor contained 50 mM Hepes (pH 7.0), 150 mM NaCl, 0.1%:0.1%:0.02% DDM:CHAPS:CHS, 2 mg/ml BSA. For CXCR4 the buffer was additionally supplemented with 5 mM $MgCl_2$ and 1 mM $CaCl_2$. The assays were performed at 25 ºC.

CCR5 and CXCR4 Detergent screen:

Detergents from a Master Solution kit (Anatrace) were plated in 96 well 1 ml plates and then aliquoted into 96 well (300 µL) plates. The plates were sealed with thermosealer and stored at 4 °C until further use.

Cells expressing CCR5 or CXCR4 (20 million cells) were suspended in 15 mL of solubilisation buffer (CCR5: 20 mM Tris HCl (pH 7.0), 100 mM $(NH_4)SO_4$, 10% glycerol; CXCR4: 20 mM Tris HCl (pH 7.0), 100 mM $(NH_4)SO_4$, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 10% glycerol) containing protease inhibitor and sonicated. For the initial screen 12 µL of a mixture containing lipids and detergents at 1:1 ratio were added to each well of a 300 µl 96 well plate. The sonicated cell suspension (120 µL) was added to each well. The plates were incubated at 4 ºC for 6 hours on the rocker. The samples were spun at 4 ºC at 3,500 rpm for 10 minutes and the supernatant was transferred into the assay plate. For the controls $6x10^6$ cells were suspended in 1 ml of standard solubilisation buffer containing protease inhibitor and the standard detergent lipid mix (CHS/DDM/CHAPS : lipids at 1:1 or 1:2 ratio for CCR5 and CXCR4 receptors respectively)

and incubated for 2 hours. The screen for CCR5 was performed in 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.1:0.1:0.02% DDM:CHAPS:CHS, 2 mg/ml BSA 25 °C. For CXCR4, 5 mM $MgCl_2$ and 1 mM $CaCl_2$ was added to the same buffer. The samples were kept at 6 °C during the screen. Each cycle included the capture of receptor followed by injection of a conformation dependent antibody (2D7 or 12G5) and regeneration solution 10 mM NaOH/ 1% n-octyl-b-D-glucopyranoside using the same injection parameters as described above for the solubilisation buffer screen. For the second screen, the same procedure was performed using the detergent identified from the first screen by including it in all conditions in a ratio of 1 detergent from screen 1: 1 variable detergent:1: lipid (18 µL total volume). Similarly the third detergent screen contained a combination of 3 detergents in 1:1:1 ratio (2 known detergents with 1 variable detergent, 24 µL total volume including 6 µL of lipids). Injection of the receptor solubilised under the standard conditions was included at the beginning, middle and end of the screen as a control. Additionally control samples of receptors solubilised using detergents identified in screens 1 and 2 were also added. These controls contained blank additions of buffer to mimic the dilution factor of the extra detergent used for screen and served as a control for the identification of any dilution effect on receptor activity caused by adding the volume of detergent to the receptor mixture. The composition of samples and controls is detailed in supplementary tables 2 and 3.

Verification of the solubilisation conditions with conformation dependent antibodies.

Detergents showing the best levels of activities for solubilisation of CCR5 and CXCR4 were selected and a series of different ratios of detergent mixtures with lipids were tested for correctly folded protein using the conformation dependent antibodies 2D7 and 12G5 as probes. Receptors were solubilised and screened using the methods described above. Detergent combinations are summarised in supplementary tables 5 and 6. A total of 64 and 58 combinations were tested for CXCR4 and CCR5, respectively.

Confirmation of active folded protein by small molecule binding:

For CXCR4, the detergent and buffer screen conditions were tested with the It1t inhibitor. CXCR4 was captured on 1D4 sensor surface in running buffer 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.1:0.1:0.02% DDM:CHAPS:CHS, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% DMSO at 25 °C. It1t was injected at concentrations 0.09 – 200 nM and 3 fold dilutions, at a flow rate of 30 μL/min, with a 1 min association and up to 5 min dissociation from lowest to highest concentrations. For CCR5, Maraviroc was used as a screening compound. CCR5 was captured on 1D4 surface in running buffer 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.1:0.1:0.02% DDM:CHAPS:CHS, 0.5% DMSO at temperature 25 °C. Maraviroc was injected at concentrations 0.004 – 10 uM and 3 fold dilutions, at a flow rate pf 30 μL/min, with a 1 min association and up to 5 min dissociation from lowest to highest concentrations.

Data analysis

The capture levels (just before 2D7 or 12G5 injection) and antibody binding levels (just after the end of 2D7 or 12G5 injection) were collected for the receptors under all conditions and the ratio of antibody:capture was calculated. The values of the ratio for each condition were normalised for the values of control conditions derived from values for controls injected at the beginning, middle and end of the screen for each flow cell separately. We used a trend of activity (ratio) decrease specific for each screen. Details of the normalisation procedures are shown in Supplementary Figure 1. The capture levels for each condition were normalised for the closest control capture level in each screen. For the CCR5 receptor the control sample injected at the beginning and end of each cycle exhibited similar activity levels showing that the chip was suitable for multiple cycles but also that the receptor is stable over time. During the CXCR4 receptor screen, in which the sample controls were screened at the beginning, middle and end, a decline throughout the screen is seen probably due to the regeneration cycles and loss of activity of 1D4 antibody surface. Thus, for data analysis, the conditions chosen for further analysis were compared to the control samples and the loss of activity was

taken into consideration. It is important to note that all data for the conformation antibody binding level to the captured receptor were normalised for capture levels as well as adjusted to levels of controls used during the screens. Normalisation for capture levels consist of calculating the ratio between the antibody binding response and the response obtained after injection of the solubilised receptor. In this case all the antibody levels are normalised in such a way that we estimate the same capture level for each solubilised receptor. This can however result in 'false positives' where for extremely low captures we can monitor a low level of conformation dependent antibody binding, and the resulting ratio will be high. To avoid this, we eliminated all conditions with significantly low captures and significantly low antibody binding levels (below 20 RU) and resulting data for these conditions were assigned to a zero level. All data collected for small molecule binding were normalised for capture levels of 3000 RU for each receptor for comparability. For data analysis and figure preparation Scrubber 2 (Biologic software), Sierra Sensors Analyser 2, Microsoft Excel and OriginPRO were used. The binding kinetics was fitted using 1:1 binding model using Scrubber 2 software.

RESULTS:

Solubilisation buffer screen:

A collection of 104 solubilisation buffers were tested for CCR5 and CXCR4 receptors' capture and activity levels. Capture levels correspond to ability of each condition to remove the receptor from the membrane, whereas the activity levels represent the level of correct folding into an active conformation and is measured by binding of conformation dependent antibody. These conditions investigate the effects of buffer components, salts, precipitants and polyols on the activity of the receptor. The list includes different molecular weights and concentrations of PEGs, a high concentration of magnesium sulphate or ammonium sulphate, 2-propanol, glycerol or sucrose and different salts covering a range of metal ions. The pH of all buffers

was kept constant at pH 7.0, the same as the control conditions. The set of buffers was designed based on availability from commercial suppliers. These included Reagent Formulations from Hampton Research (MembFac HT (HR2-137 Reagent Formulation), Crystal Screen (HR2-110 Reagent Formulation) and PEG/Ion Screen (HR2-126 Reagent Formulation)). The list of solubilisation buffers used is shown in Supplementary Table 1.

The folding of the receptors into an active conformation, which we refer to as receptor activity, was monitored by the binding of the conformation specific antibodies 2D7 and 12G5 for CCR5 and CXCR4, respectively. The normalised antibody:capture ratios were plotted against the solubilisation buffers conditions. To simplify the presentation of the screen the buffers and additives were combined into related groups that served for construction of 3D graphs for the solubilisation buffer screen. The graphs are shown in Figure 1 A for CCR5 and B for CXCR4. The binding of the antibody to control samples is represented by a plane at response level 1 with transparent planes above and below representing the standard deviation. Bars showing the responses above the control correspond to solubilisation conditions that improve the activity of receptors and bars below the control response level representbuffer conditions less suitable for maintaining receptor in an active conformation. A similar approach was used to analyse the capture levels for each solubilisation buffer. The resulting 3D plots are shown in figure 1 C and D. The following results of the solubilisation buffer screen focuses on the influence of the buffer additives separately on each receptor activity (e.g. foldedness into an active conformation) and capture levels (absolute amount of receptor captured).
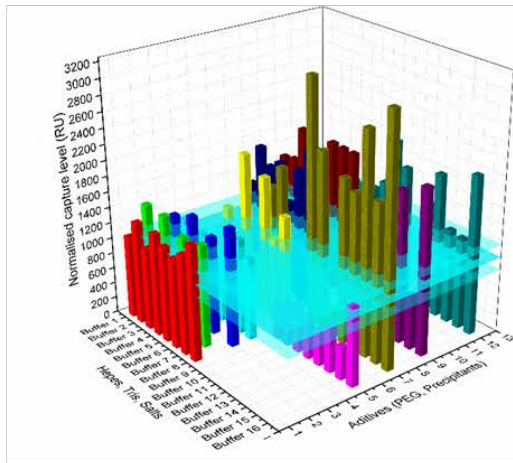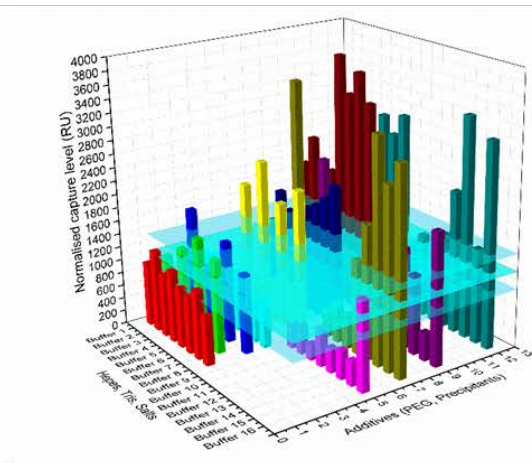
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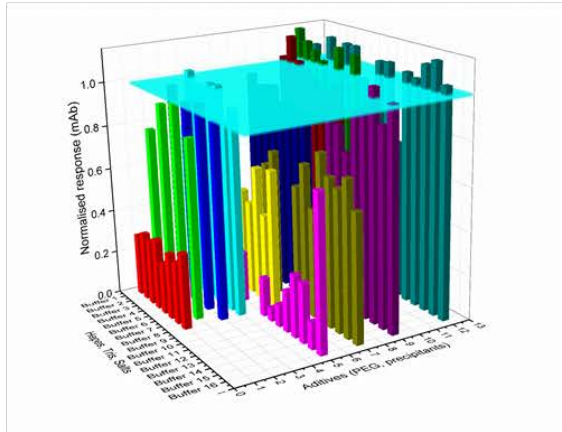
B
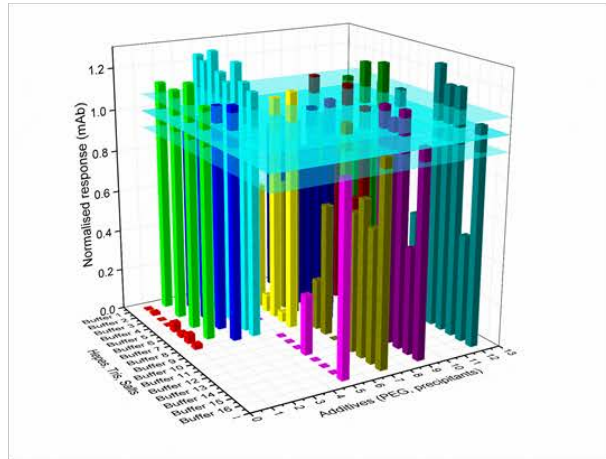
C

D

Solubilisation buffer graph legend BUFFERS	
Buffer 1	100 mM Hepes+100 mM NaCl+10% glycerol
Buffer 2	100 mM Hepes+100 mM Ammonium Suplhate+10% glycerol
Buffer 3	100 mM Hepes+100 mM NaCl+10% sucrose
Buffer 4	100 mM Hepes+100 mM Ammonium Suplhate+10% sucrose
Buffer 5	100 mM TrisHCl+100 mM NaCl+10% glycerol
Buffer 6	100 mM TrisHCl+100 mM Ammonium Suplhate+10% glycerol
Buffer 7	100 mM TrisHCl+100 mM NaCl+10% sucrose
Buffer 8	100 mM TrisHCl+100 mM Ammonium Suplhate+10% sucrose
Buffer 9	100 mM TrisHCl+100 ml Calcium chloride dihydrate+10% glycerol
Buffer 10	100 mM TrisHCl+100 mM Magnesium chloride hexahydrate+10% glycerol
Buffer 11	100 mM TrisHCl+100 mM Zinc Acetate dihydrate+10% glycerol
Buffer 12	100 mM TrisHCl+100 mM Potassium phosphate monobasic+10% glycerol
Buffer 13	100 mM TrisHC+ 100 mM Sodium citrate tribasic dihydrate+10% glycerol
Buffer 14	100 mM TrisHCl+Lithium sulphate monohydrate+10% glycerol
Buffer 15	100 mM Tris HCl + 10% glycerol
Buffer 16	100 mM Tris HCl + 100 mM Ammonium Sulphate

Solubilisation buffer graph legend ADDITIVES	
1	10% 2-propanol
2	1M Ammonium Sulphate
3	No additives
4	1M Magnesium sulphate hydrate
5	20% PEG 8000
6	15% PEG 8000
7	12% PEG 6000
8	10% PEG 6000
9	15% PEG 4000
10	10% PEG 4000
11	15% PEG 400
12	15% PEG 1500

Figure 1. A. Normalised binding levels for the 2D7 antibody binding to the CCR5 receptor solubilised using 102 different solubilisation buffer conditions. The light blue plane represents the binding level of 2D7 antibody with standard deviation for CCR5 solubilised using the standard control condition. B. Normalised binding levels for 12G5 antibody binding to CXCR4 receptor

solubilised using 102 different solubilisation buffer conditions. The light blue plane represents the binding level of 12G5 antibody with standard deviation for CXCR4 solubilised using the standard control condition. The buffers 1-16 and additives 1-12 are summarised in the tables. C. Normalised capture levels for the CCR5 receptor solubilised using 102 different solubilisation buffer conditions. The light blue plane represents the capture level with standard deviation for CCR5 solubilised using a standard control condition. D. The normalised capture levels for CXCR4 receptor solubilised using 102 different solubilisation buffer conditions. The light blue plane represents the capture level with standard deviation for CXCR4 solubilised using a standard control condition. Buffers 1-16 and additives 1-12 are summarised in the tables.

In the presence of a consistent mix of DDM/CHS/CHAPS/lipids each set of buffers was screened utilising either 100 mM NaCl or 100 mM $(NH_4)_2SO_4$ as the salt. Substitution of ammonium sulphate for NaCl without other additives e.g. buffer 1 compared with buffer 2 etc. has little effect on the activity with similar values seen in each corresponding condition suggesting that it is the addition of other additives that can have more of an effect. The addition of 1 M ammonium sulphate (Additive 2, Fig1 A and B) has slightly more of an effect on the activity with CCR5 activity generally slightly lower than the control condition and the activity of CXCR4 generally slightly higher, the capture is either a bit higher or close to the control for both CXCR4 and CCR5. In contrast the presence of 2-propanol in solubilisation buffers has a negative effect on active folding. Very low or no activity was observed for CXCR4 receptor in all the solubilisation buffers tested while for the CCR5 receptor the activity was only about 50% of the control. Interestingly, the capture levels were similar for both CCR5 and CXCR4 receptor at levels 800 – 1200 RU suggesting that while the amount of protein captured and thus solubilised is similar the 2-propanol had more of an effect on the folding of CXCR4 into an active conformation than CCR5. Magnesium sulphate (additive 4) addition in buffers 1-8 solubilises CCR5 at activities close to control activity with capture levels that are similar to the control. For CXCR4 the same buffers show a higher activity of receptor in the presence of magnesium sulphate however the capture levels of CXCR4 relative to the CXCR4 control are lower. Generally, the addition of 20% PEG 8000 both lowers activity (foldedness) and also capture (solubilisation) for the CCR5 receptor in all the solubilisation buffers tested (buffers 5-6 and 9-16). In contrast to the buffers containing metal ions, the presence of ammonium

sulphate slightly, particularly in the absence of glycerol, increases the activity when compared to the other buffers in the presence of 20% PEG 8000. Interestingly, the condition without glycerol has a capture level similar to the control and an activity 78% of the control suggesting the combination of PEG8000 with glycerol has a negative effect on both solubilisation and obtaining an active conformation. A similar pattern of low activity is seen when the concentration of PEG 8000 is 15%, however for most of the buffers tested at this concentration (1-8) the activity is higher than at 20% PEG 8000 and the solubilisation is better thus confirming that the PEG 8000 is having a negative effect. For CXCR4 the pattern is different. At a concentration of 20% PEG, in common with CCR5 there is generally low capture, with the exception of the buffer containing no glycerol (buffer 16). Additionally, there is no activity apart from buffer 16 (close to control levels) and the phosphate containing buffer 12 (54% of the control). At 15% PEG 8000 we observe higher activity in buffers containing ammonium sulphate compared to NaCl as well as a dramatic increase in capture levels. The increase of PEG 8000 concentration from 15% to 20%, in buffers 5 and 6, significantly reduces activity.

10% PEG 6000 provides similar good levels of activity and high capture levels for CCR5 with either ammonium sulphate or NaCl (buffers 1-8). An increase in PEG 6000 concentration to 12% coupled with the addition of different metal ions (buffers 9-10 and 12-16), with the exception of buffer 11 containing zinc acetate, results in high or very high capture levels (solubilisation) but lower activity levels than in buffers 1 to 8. Buffer 11 (zinc acetate) not only has the lowest activity level (53% of the control) but it also displays poor solubilisation (capture). CXCR4 behaves similarly to CCR5 in buffers 1-8 with good activity levels for all buffers. However, buffers 2, 4, 6, 8 and 16 containing ammonium sulphate consistently have higher activity levels than the equivalent NaCl containing buffers. With the exception of buffer 12 containing potassium phosphate, the activity in the presence of 12% PEG 6000 with different metal and anions is lower than the control. In particular, very low activity was observed for buffer 9 with calcium chloride and no activity for buffer 11 (zinc acetate). Capture

levels (solubilisation) were generally high except for buffers 9-11 containing calcium, magnesium and zinc.

Buffers containing 10 or 15 % PEG 4000 generally show good activity and good levels of capture for CCR5. The exception is a very low capture level with moderate activity observed for zinc acetate containing buffer 11 (15% PEG 4000). For CXCR4 PEG 4000 maintains a level of activity similar to the control for buffers 2, 4, 6, 8 and thus are similar to the PEG 6000 conditions where ammonium sulphate containing buffers are more active than the equivalent buffers with NaCl or no salt (buffer 15). Buffers 12, 13, 14 comprising potassium phosphate, sodium citrate or lithium sulphate also give high levels of activity and capture. The absence of glycerol (buffer 16) has no effect on activity. This contrasts with the very low captures and the low or no activity that were observed for buffers 9-11 containing calcium, magnesium and zinc.

15 % PEG 1500 shows good activity (better than the control in a number of conditions) for CCR5 with high capture levels except in buffer 11 containing zinc where the activity is also lower. For CXCR4 the activity is high (similar or better than the control) except in buffers 9-11 and 15 containing calcium, magnesium, zinc and no salt. The activity of the ammonium sulphate containing buffer was consistently higher than equivalent NaCl buffer. All tested buffers showed high capture levels except buffer 11 containing zinc.

PEG 400 maintains good activity for all tested buffers for both CCR5 and CXCR4. Capture levels are higher for CCR5 compared to CXCR4 with the level of capture of CXCR4 consistently at about 50% of the control. Despite the lower capture levels for CXCR4, the same pattern of higher activity (close to or higher than the control level) seen in PEG/ammonium sulphate containing buffers compared with the equivalent PEG/NaCl buffers is observed. For CCR5 a number of conditions have a higher activity than the control.

We did not observe significant difference on receptor activity between equivalent glycerol and sucrose conditions, although the majority of screened conditions did show slightly better

activity in the presence of glycerol. A selection of 32 buffers was also designed to compare the presence of Tris or Hepes. Again, although we did not see significant differences in activity for CCR5, CXCR4 shows a slight preference for Tris based solubilisation buffers compared to Hepes.

In summary, the activity of CXCR4 was negatively affected by buffers containing calcium chloride, magnesium chloride and zinc acetate (buffers 9-11) or isopropanol. The addition of 1M magnesium sulphate gave the highest levels of activity with ammonium sulphate having a similar positive effect. Buffers containing 15% PEG 400 in combination with 100 mM ammonium sulphate or PEG 1500 with potassium phosphate, sodium citrate or lithium sulphate also gave levels of activity higher than the control. For CCR5 there was less of a dramatic effect however a number of conditions containing either PEG 1500 or PEG 400 result in activity levels higher than the control. Interestingly in the top 20 solubilisation conditions for both receptor, seven conditions (Table 1 Supplementary data; 16, 26, 42, 80, 84, 88 and 89) overlap for both CCR5 and CXCR4 containing mainly 15% PEG 1500 or 4000 and the presence of sulphates.

Solubilisation buffer confirmation of active folding by binding of small molecules

Twenty solubilisation buffers (2, 4, 10, 11, 12, 16, 19, 26, 27, 42, 50, 51, 68, 72, 80, 84, 88, 95, 99, 100) were further selected for CCR5 and tested for binding of the slow off-rate small molecule maraviroc [16]. For better comparison of the conditions, binding sensorgrams for single concentration 370 nM are shown in Figure 2 – data collected on Sierra Sensors MASS-1 and Biacore T200 instruments. In the graph next to each sensorgram the overlay shows binding response at this concentration. All responses were normalised for a capture level of 3000 RU. The highest response was observed in condition 99 containing ammonium sulphate and PEG 1500 (Supplementary Table 1).
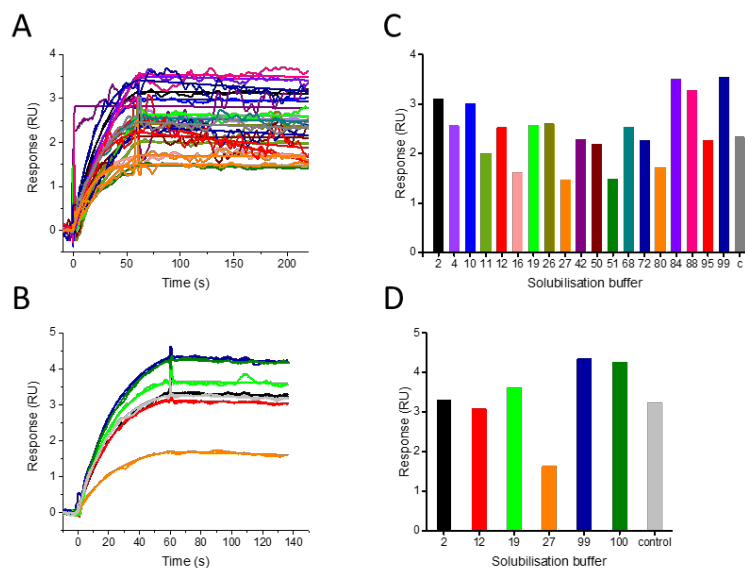
Figure 2: Binding responses for maraviroc injected at 370 nM to CCR5 solubilised at different conditions collected on MASS-1 (A) and Biacore T200 (B) instruments. Smooth traces correspond to 1:1 kinetic fit. C, D: Comparison of the binding levels for each tested solubilisation condition. The response level is read from each sensorgram just after end of the injection. The colours of the bars correspond to the colours of sensorgrams, the number on the x axis corresponds to solubilisation buffer ID (Supplementary table 1).

Due to the lower number of detected conditions showing a better activity for CXCR4 compared with the control, we selected only seven buffer conditions (8, 26, 27, 32, 56, 80 and 88) based on 12G5 levels as well as variability of additives in buffer composition and tested these using Sierra sensors MASS-1 and Biacore T200 instruments. CXCR4 inhibitor It1t at concentration series 0.4 – 200 nM was injected over the surfaces. Sensorgrams are shown in figure 3 (A: Data collected using MASS-1, B: data collected using Biacore T200). Buffers 56, 80 and 88 containing magnesium sulphate, and potassium phosphate and lithium sulphate with addition of PEG 1500 showed the best response levels compared to the control solubilisation condition. The kinetic parameters are summarised in Table 1.
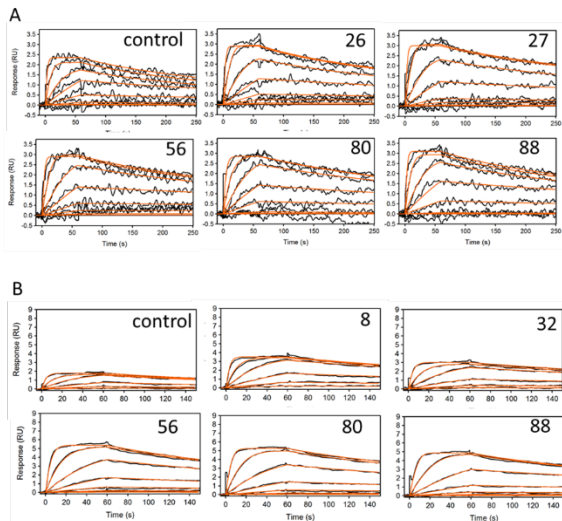
Figure 3. Binding sensorgrams for It1t bound to CXCR4. It1t was injected at concentrations 0.09 to 200 nM. A. the binding to CXCR4 receptor solubilised using 6 different solubilisation buffers: control, 26, 27, 56, 80 and 88 was analysed using the MASS-1 instrument. B. binding to CXCR4 receptor solubilised using 6 different solubilisation buffers: control, 8, 32, 56, 80 and 88 was analysed using the Biacore T200. The orange traces represent a 1:1 kinetic fit. The buffer ID composition is summarised in Supplementary table 1.

Table 1: Kinetic parameters for It1t compound binding to CXCR4 solubilised using different solubilisation buffers and detergents (Solubilisation buffer – Supplementary Table 1, Detergent used for solubilisation, Detergent present in the assay buffer, kinetic parameter of binding – k_a – on-rate, k_d – off-rate and K_D – affinity, corresponding figure).

Instrument	Solubilisation buffer	Detergent in solubilisation buffer	Detergent in assay buffer	k_a (M^{-1}s^{-1})	k_d (s^{-1})	K_D (nM)	
Mass-1	Standard buffer	CDC	CDC	2.95(±0.08)e6	0.0036(±0.0005)	1.21(±0.04) nM	
	26	CDC	CDC	3.08(±0.08)e6	0.0035(±0.0005)	1.13(±0.03) nM	
	27	CDC	CDC	3.4(±0.1)e6	0.0028(±0.0005)	0.83(±0.03) nM	
	56	CDC	CDC	2.73(±0.05)e6	0.0030(±0.0003)	1.10(±0.02) nM	
	80	CDC	CDC	1.56(±0.03)e6	0.0025(±0.0002)	1.58(±0.03) nM	
	88	CDC	CDC	3.01(±0.07)e6	0.0030(±0.0003)	1.01(±0.03) nM	Fig.3
T200	Standard buffer	CDC	CDC	1.39(±0.02)e6	0.0044 (±0.0004)	3.24(±0.06) nM	
	8	CDC	CDC	1.39(±0.01)e6	0.0032(±0.0001)	2.28(±0.02)nM	
	32	CDC	CDC	1.51(±0.02)e6	0.0032(±0.0002)	2.14(±0.02)nM	
	56	CDC	CDC	1.32(±0.06)e6	0.0043(±0.0001)	3.27(±0.01)nM	
	80	CDC	CDC	1.69(±0.02)e6	0.0043(±0.0002)	2.53(0.02)nM	

	88	CDC	CDC	1.64($\pm$0.01)e6	0.0048($\pm$0.0002)	2.95($\pm$0.01)nM	
	Standard buffer	CDC	CDC	9.02($\pm$0.07)e5	0.0033($\pm$0.0001)	3.69($\pm$0.02)nM	
	Standard buffer	58	CDC	9.60($\pm$0. 10)e5	0.0036($\pm$0.0002)	3.69($\pm$0.03)nM	
	Standard buffer	59	CDC	1.08($\pm$0.06)e6	0.0038($\pm$0.0001)	3.54($\pm$0.02)nM	
	56	59	CDC	1.47($\pm$0.01)e6	0.0048($\pm$0.0002)	3.25($\pm$0.02)nM	Fig.5
	80	59	CDC	1.28($\pm$0.01)e6	0.0045($\pm$0.0002)	3.55($\pm$0.03)nM	
	Standard buffer	59	0.1% T323 + 0.05% B310	1.74($\pm$0.02)e6	0.0050($\pm$0.0002)	2.85($\pm$0.02)nM	
	56	59	0.1% T323 + 0.05% B310	1.63($\pm$0.02)e6	0.0052($\pm$0.0003)	3.16($\pm$0.03)nM	
	80	59	0.1% T323 + 0.05% B310	1.79($\pm$0.02)e6	0.0056($\pm$0.0003)	3.15($\pm$0.03)nM	

Detergent screens for CCR5 and CXCR4 receptors

Detergents represent an essential component for both solubilisation and the activity of membrane proteins. The importance of selecting correct detergents was reported previously [9, 11]. We used the Biacore 4000 and 3000 instruments to screen 99 different detergents for receptor solubilisation and a total of over 300 mixtures of the detergents to determine which combinations can improve or maintain receptor activity for binding of the conformation dependent antibodies 12G5 and 2D7. We found both CCR5 and CXCR4 receptors prefer different detergents for maximal activity but also can maintain activity using the same detergents. The detergent mixtures were identified by systematic screen of series of 99 detergents followed by mixing the best detergent with the remaining detergents for follow up screens as described previously [17]. In the first detergent screen we found the best solubilisation conditions for CCR5 were with using MNG, then maltosides, CHAPS/CHAPSO, cymal-4, GDN and sucrose monododecanoate (Figure 4A,C; Supplementary data Table 3). For the second screen MNG detergent was mixed with each of the rest of detergents in a ratio 1:1. Again, solubilisation conditions having GDN, several maltosides and sucrose monododecanoate showed high responses for conformation dependent antibody. Interestingly, some conditions such as anapoe detergents, that showed minimal or no binding

in the first screen, showed high responses. This can be caused by complementing the efficiency of the second detergent used in the mixture. The third screen contained mixtures of MNG+GDN in all detergent conditions. PMAL-C8 showed highest response in combination with MNG and GDN followed by tetradecyl-B-D-maltoside. Several maltosides and also sucrose monododecanoate showed high responses in this detergent mixture.

The first detergent screen for CXCR4 receptor showed highest responses for MNG and tridecyl-b-D-maltoside. Several other maltosides, cymal-4, sucrose monododecanoate, Chaps, Chapso and Big CHAP deoxy also solubilised CXCR4 receptor at high activities. For the second screen tridecyl-b-D-maltoside was added to mixtures of all detergents. Highest responses were observed for mixtures containing Big CHAP and Big CHAP deoxy. The third screen contained the mixture of screening detergents with tridecyl-b-D-maltoside and Big CHAP. The highest response with this mixture was in the presence of GDN, however the response was lower compared to mixture of just two detergents from previous screen (tridecyl-b-D-maltoside and Big CHAP). Capture levels for each detergent mixture are shown in Supplementary Table 2 and Figure 4 B, D. Interestingly, the highest capture levels show low or no binding to the conformation dependent antibody. Capture levels can provide an indication of efficacy of the solubilisation condition for the receptor but do not indicate how active the receptor is. Indeed the highest receptor activity was observed for conditions where CXCR4 was captured at intermediate levels comparable with control (~500-800 RU). It is likely that the low activity of CXCR4 captured at high levels is caused by CXCR4 forming aggregates and capturing non-specifically to the surface. However, low capture levels do suggest low a solubilisation efficiency that is not suitable for extraction of receptor from the membrane. For CCR5 the normalised intermediate capture levels for the active receptor were at higher levels compared to CXCR4 1500-2000 RU. Supplementary Table 4 summarises detergents showing top 10 highest activity levels for both CCR5 and CXCR4. We found approximately 70% overlap of detergents suitable for both receptors. MNG detergent that is a common detergent used for GPCRs purification and assays was suitable for both receptors. Additionally, we found n-

dodecyl-B-D-thiomaltoside provided very good activity for both receptors (ratios for CXCR4 = 0.93 and CCR5 0.63) and was found more suitable than n-dodecyl beta maltoside (DDM; (ratios for CXCR4 = 0.84 and CCR5 0.61) commonly used for GPCR preparations.

Verification of detergent mixtures with conformation dependent antibodies:

To further confirm which detergent mixtures improve the activity of CXCR4, we selected 3 detergents: tridecyl-β-D maltoside, Big CHAP and MNG and prepared various mixtures of the detergents with lipids for screening of the CXCR4 activity. Supplementary data Table 6 and Figure 4F summarises the concentrations of each detergent in mixture and the activity in each mixture. It is interesting to see that various combinations of detergents and lipids at different concentrations can significantly influence activity of solubilised receptor. We found the combination of MNG and lipids (ranking condition 58, 57, 56 and 52) and T323, B310 and lipids (condition 59) showed the best results (ratio values 1.20-1.33). Generally, the presence of lipids slightly increases the activity of the receptor and increasing detergent concentration has negative effect on receptor activity. For CCR5 we selected MNG, GDN, n-tetradecyl-β-D-maltoside (T315) and Anapoe-35 detergents in mixtures with lipids. None of the tested detergent mixtures showed activity above the control and therefore were not pursued further (Supplementary data Table 5 and Figure 4E). The highest activity was seen with combinations of detergents MNG, Anapoe, GDN and lipids. Higher detergent concentrations also significantly decreased receptor activity.
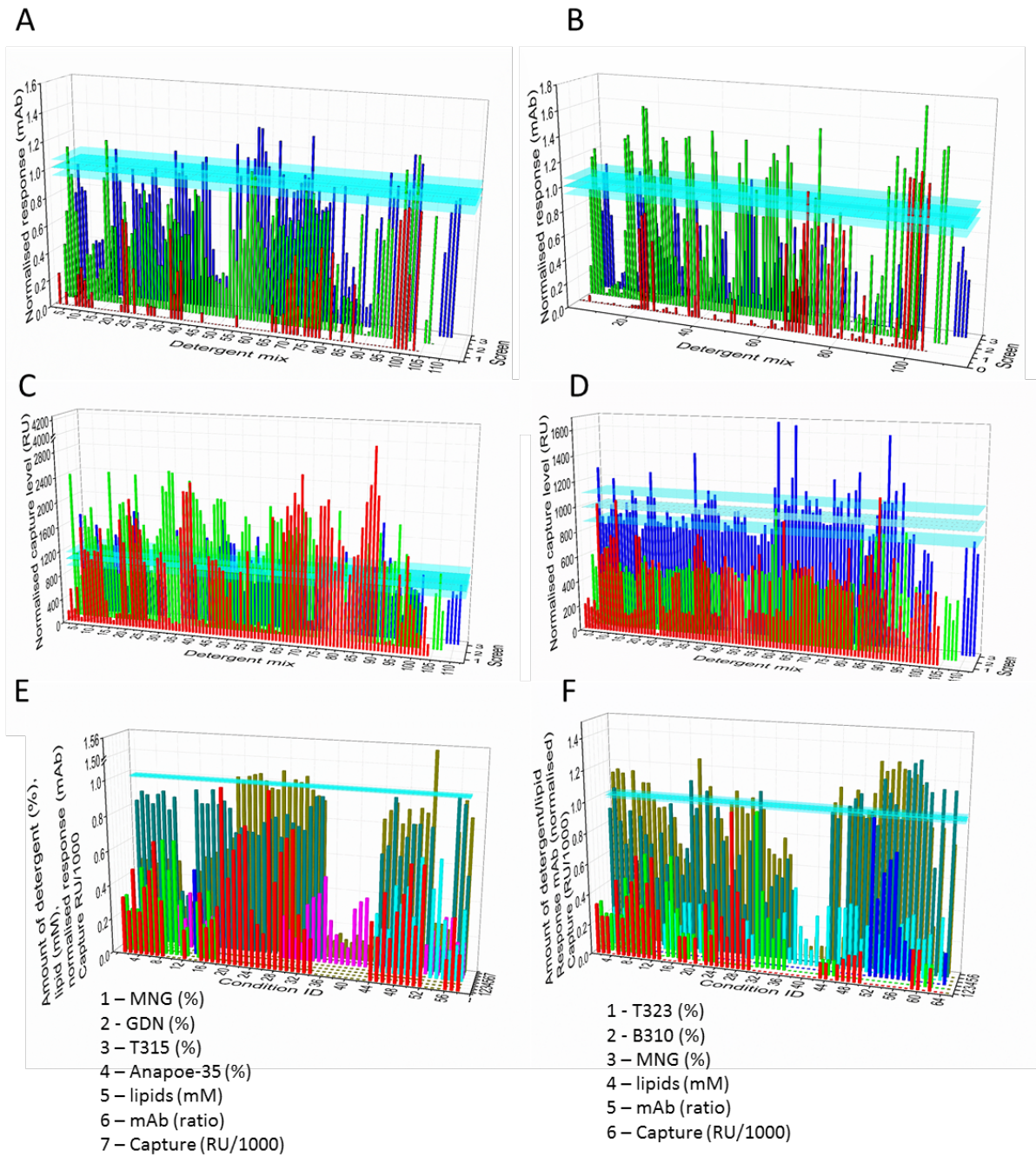
Figure 4. A. Normalised binding level responses for 2D7 antibody binding to CCR5 solubilised using different detergent conditions for screens 1-3. The light blue plane represents the binding level of the 2D7 antibody with standard deviation for CCR5 solubilised using a standard control condition. B. Normalised binding level responses for 12G5 antibody binding to CXCR4 solubilised using different detergent conditions for screens 1-3. The light blue plane represents the binding level of the 12G5 antibody with standard deviation for CXCR4 solubilised using a standard control condition. C. Normalised capture levels for CCR5 receptor solubilised using different detergent conditions. The light blue plane represents the capture level with standard deviation for CCR5 solubilised using a standard control condition. D. Normalised capture levels for the CXCR4 receptor solubilised using different solubilisation

detergent conditions. The light blue plane represents capture level with standard deviation for CXCR4 solubilised under a standard control condition. E. Normalised binding level responses for the 2D7 antibody binding to CCR5 solubilised in different detergent/lipid mixtures and capture levels. The light blue plane represents binding level of 2D7 antibody with standard deviation for CCR5 solubilised using the standard control condition. F. Normalised binding level responses for the 12G5 antibody binding to CXCR4 solubilised in different detergent/lipid mixtures and capture levels. The light blue plane represents binding level of 12G5 antibody with standard deviation for CXCR4 solubilised using the standard control condition

Verification of detergent solubilised protein with small molecules:

To further determine the effect of detergents on activity of solubilised receptors we measured binding of small molecules It1t and maraviroc to CXCR4 and CCR5 receptors respectively, using the detergent conditions that showed highest binding responses to the conformation dependent antibodies. For CXCR4 we compared detergent mixtures 58 (MNG) and 59 (T323, B310) (Figure 5 A-H, Supplementary table 6). We found detergent 59 slightly improved binding activity of the receptor over the control and this improvement was enhanced by adding this detergent mixture into the assay buffer. Solubilisation condition 58 showed lower response for compound binding compared to the control. For CCR5 we tested detergent mixtures 15 (MNG and GDN) and 34 (MNG) as these detergents were selected for the main detergent screen as well as ranked high in the confirmation screen. We found the control showed best binding affinity. Interestingly the binding in condition 15 could be improved by replacing the standard detergent in the assay buffer with 0.01% MNG – which is one component of the detergent mixture used for solubilisation (Figure 5 I). Condition 58 for CXCR4 and 34 for CCR5 are identical, interestingly both these conditions decrease the activity for both receptors to ~ 50% compared to control even though the conformation antibody binding is amongst the best compared to other tested detergent mixtures.
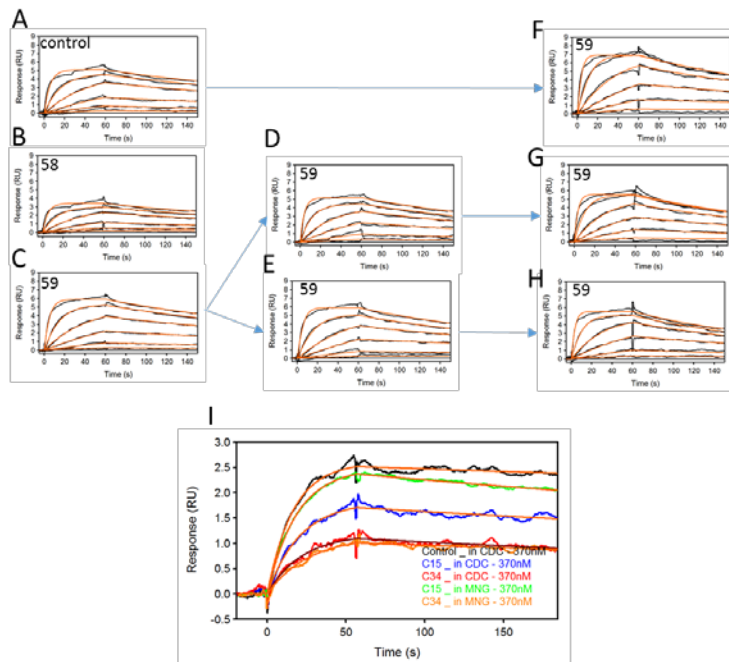
Figure 5. A-H. Binding sensorgrams for It1t bound to CXCR4. It1t injected at concentrations 0.09 to 200 nM binding to CXCR4 receptor solubilised using different detergent mixtures (supplementary table 6) and solubilisation buffers (supplementary table 1). A. Control solubilisation condition, B. CXCR4 solubilised using mixture 58 and standard solubilisation buffer. C. CXCR4 solubilised using mixture 59 and standard solubilisation buffer. D. CXCR4 solubilised using detergent mixture 59 and solubilisation buffer 56. E. CXCR4 solubilised using detergent mixture 59 and solubilisation buffer 80. F. CXCR4 solubilised using detergent mixture 59, standard solubilisation buffer and assay buffer containing detergent mixture 0.1% T323 + 0.05% B310. G. CXCR4 solubilised using detergent mixture 59, solubilisation buffer 56 and assay buffer containing detergent mixture 0.1% T323 + 0.05% B310. H. CXCR4 solubilised using detergent mixture 59, solubilisation buffer 80 and assay buffer containing detergent mixture 0.1% T323 + 0.05% B310. I. Binding sensorgrams for maraviroc injected at 370 nM to CCR5 solubilised using different detergent combinations (supplementary table 5). Black-CCR5 control (solubilisation-CDC (CHS/DDM/CHAPS), assay buffer – CDC), green (solubilisation-15, assay buffer – MNG), blue (solubilisation-15, assay buffer – CDC), red (solubilisation-34, assay buffer – CDC), orange (solubilisation-34, assay buffer – 0.01% MNG)

Comparison of Screening Instruments

The application of several SPR platforms for screening and verifying solubilisation conditions improved the assay throughput and also enabled a comparison of these platforms. The general approach was the same for all the instruments, where the 1D4 antibody was immobilised over all flow cells (and spots with respect to Biacore 4000 experiments) and the solubilised samples were captured via the C9 tag followed by conformational antibody injection and finally regeneration of the antibody surface enabling the performance of the next cycle using the same antibody surface. Using the Biacore 4000 which is capable of processing 4

samples simultaneously and having 5 detection spots, meant that the methods benefited from higher throughput and more samples could be processed per cycle.

When using the Biacore 3000 one sample is processed per injection. It was possible to capture three solubilised receptors per cycle. Solubilised receptor was captured over flow cell 1, 3 and 4, leaving flow cell 2 as a reference flow cell. The rest of the injections were the same as the Biacore 4000 experiment. In the setup for the Biacore 3000 the methods were longer and also the number of the regeneration cycles increased for each run thus a lower number of samples could be processed per cycle. Although, one sensor chip was sufficient for the study of ~ 100 solubilisation conditions on the Biacore 4000, on the Biacore 3000 two sensor chips were needed for the screening as the antibody surface lost activity after multiple regeneration cycles. However, the buffer and reagent consumption was significantly lower on Biacore 3000 compared to the Biacore 4000. Thus, the major difference is the choice between a time- or cost-efficient platforms.

The Sierra Sensors MASS-1 instrument benefits from the simultaneous processing of 8 samples in one injection where samples are addressed over 8 separate channels containing one reference and one target spot. The advantage of this instrument is in its ability to rapidly screen a large variety of solubilisation conditions while providing reasonable sensitivity for small molecule binding. We used this technology to confirm selected solubilisation buffer conditions for CXCR4 and CCR5 receptors using small molecules It1t and Maraviroc respectively injected at 8 concentrations each. One sensor chip was suitable to test up to 24 conditions within 3 days (compared to 3 conditions/day using Biacore T200) providing a time-effective approach to confirmation of the solubilisation buffers.

The MASS-1 instrument data quality was comparable to Biacore T200. Both data collected for CXCR4 and CCR5 on the Biacore T200 and Sierra MASS-1 instrument were at low level of response units however the kinetic and affinity values collected on both instruments were in the same range for all screened solubilisation conditions.

When using different instruments for the assays it is important to include controls within each screen to maintain comparability of the results. For example as seen in Fig. 2 C and D the ranking of solubilisation buffer conditions for CCR5 receptor interaction with maraviroc is very similar for both instruments. Although the condition 19 shows higher response compared to condition 2 in dataset collected on Mass-1 instrument compared to data obtained from T200 instrument, this was the only discrepancy found. However condition 19 did not provide the best solubilisation activity for binding of small molecule maraviroc from the conditions selected for confirmations. The best solubilisation buffer (condition 99) was identified by both instruments independently suggesting reasonable good comparability of assays collected with both technologies.

Discussion

In this study we used surface plasmon resonance technology to screen solubilisation conditions for two GPCRs: CXCR4 and CCR5. Both receptors belong to the chemokine family and are both involved in HIV virus entry into host cells. However, these receptors share only 30.4% identity suggesting the results from the solubilisation screens can provide information on the transferability of the solubilisation conditions between a wider varieties of GPCRs.

These screens in this paper followed on studies published previously [11, 12, 17] however at a larger scale. A total of 950 different solubilisation conditions containing various buffer components and detergent/lipid mixtures were explored to monitor which of these conditions can solubilise each receptor in an active conformation and determine the similarities and differences between the solubilisation of the two receptors. We also compared the strengths of each solubilisation condition by monitoring the capture levels of each receptor. Interestingly we found some conditions that were influencing receptor activity similarly for both GPCRs, but also some conditions had an opposite effect. We observed that conditions with high solubilisation strengths – resulting in high capture levels do not always solubilise the receptor

in an active conformation. Extremely high capture levels, well above the levels for controls, may be caused by aggregation of the receptor in a specific detergent and/or non-specific binding to the sensor surface. Very low capture levels can provide high activity levels relative to the capture level but in this case the sensitivity of the assay can then also significantly decrease. We were also able to examine the correlation with these data on active folding obtained by binding of a high molecular weight antibody in a high-throughput screen to a smaller low through-put verification screen using small molecules as probes of receptor activity. Interestingly we found that even though some solubilisation conditions showed high levels of binding to the receptor for the conformation-dependent antibody the ranking of these conditions did not translate fully to an activity-based ranking for small molecule binding. For example, for CCR5 receptor the best solubilisation buffer condition was found to be condition 2 containing 15% PEG 400 (Supplementary table 1) based on a conformation-dependent antibody response level, however this condition ranked 4th from data collected for binding of maraviroc. Maraviroc bound best in condition 99 containing 15% PEG 1500 which ranked 17th based on antibody response level. For CXCR4 receptor the correlation was better, for example the best response to 12G5 antibody to CXCR4 was found for receptor solubilised in buffer condition 56 containing Magnesium and 10% sucrose, which ranked 2nd for It1t binding response level. The best condition based on It1t binding was found to be condition 80 containing potassium and 15% PEG 1500 which ranked 4th for binding of antibody. Similar observations were recorded for detergent screens. Discrepancies between these two types of assays may be mainly caused by the assays monitoring different binding sites and also the binding mechanism for both antibody and small molecules. The ranking based on antibody binding serves mainly to distinguish between 'good' and 'bad' solubilisation conditions and are useful mainly due to their large molecular weight and therefore larger signal enabling these assays to be run in a high through-put mode. In order to obtain a higher degree of accuracy when comparing the best conditions identified by antibody screens, verification with small molecules is also necessary.

Conclusion

By using the high through-put solubilisation condition screens for the two receptors CXCR4 and CCR5 we have determined buffer and detergent components that can keep the receptors in an active form. Using the minimum activity threshold at 0.9 we found at least 51 buffer conditions for CCR5 receptor and 46 buffer conditions for CXCR4 receptor that can be used for solubilisations without any significant loss of activity. 32 conditions were amongst best conditions for both receptors suggesting ~30% overlap of tested solubilisation buffers (supplementary table 7). We also found 58 detergent combinations for CCR5 and 77 detergent combinations for CXCR4 that can maintain receptor activity above level of 0.9. These data can provide important indicators for optimising an activity assay for example CXCR4 prefers ammonium sulphate rather NaCl as a salt in the solubilisation buffer. Moreover, the selection of different solubilisation buffers and detergents is important for alternative methods to study membrane proteins that can be used in addition to or instead of SPR, such as X-ray crystallography, biochemical assays, radio-ligand assays, small molecule and fragment screens. Not all detergents and buffer components are compatible with such assays and thus having flexibility in the selection from which buffers or detergent can be chosen is advantageous.

By testing the two receptors we could see that despite being members of the same family there were both some similarities and differences in their preference for different conditions. While for these proteins that had known solubilisation conditions to produce active proteins we achieved modest increases in active solubilisation. These data do suggest the starting point for determination of a solubilisation buffer composition for an unknown protein and the variables worth testing such as type of salt, the addition of a low molecular weight PEG in order to optimise purification of active protein as well as the combination of detergents. We show an important parameter is the detergent that is selected for mixtures, in particular a detergent which looks unpromising on its own can in combination with another detergent

confer good solubilisation and activity. Thus, ideally a matrix that screens mixtures of detergents should be used to fully determine the ideal conditions for the receptors of interest.

Acknowledgement

References

1. Navratilova, I. and A.L. Hopkins, *Fragment screening by surface plasmon resonance.* ACS Med Chem Lett, 2010. **1**(1): p. 44-8.
2. Cooke, R.M., et al., *Preparation of purified GPCRs for structural studies.* Biochem Soc Trans, 2013. **41**(1): p. 185-90.
3. Errey, J.C., et al., *Purification of Stabilized GPCRs for Structural and Biophysical Analyses.* Methods Mol Biol, 2015. **1335**: p. 1-15.
4. Chen, D., et al., *Fragment screening of GPCRs using biophysical methods: identification of ligands of the adenosine A(2A) receptor with novel biological activity.* ACS Chem Biol, 2012. **7**(12): p. 2064-73.

5.   Rasmussen, S.G., et al., *Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor.* Nature, 2011. **469**(7329): p. 175-80.

6.   Jamshad, M., et al., *G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent.* Biosci Rep, 2015. **35**(2).

7.   Naranjo, A.N., et al., *Impact of purification conditions and history on A2A adenosine receptor activity: The role of CHAPS and lipids.* Protein Expr Purif, 2016. **124**: p. 62-7.

8.   Chattopadhyay, A., B.D. Rao, and M. Jafurulla, *Solubilization of G protein-coupled receptors: a convenient strategy to explore lipid-receptor interaction.* Methods Enzymol, 2015. **557**: p. 117-34.

9.   Navratilova, I., M. Dioszegi, and D.G. Myszka, *Analyzing ligand and small molecule binding activity of solubilized GPCRs using biosensor technology.* Anal Biochem, 2006. **355**(1): p. 132-9.

10.   Navratilova, I., et al., *A biosensor-based approach toward purification and crystallization of G protein-coupled receptors.* Anal Biochem, 2006. **353**(2): p. 278-83.

11.   Navratilova, I., J. Sodroski, and D.G. Myszka, *Solubilization, stabilization, and purification of chemokine receptors using biosensor technology.* Anal Biochem, 2005. **339**(2): p. 271-81.

12.   Rich, R.L., et al., *Detergent screening of a G-protein-coupled receptor using serial and array biosensor technologies.* Anal Biochem, 2009. **386**(1): p. 98-104.

13.   Babcock, G.J., et al., *Ligand binding characteristics of CXCR4 incorporated into paramagnetic proteoliposomes.* J Biol Chem, 2001. **276**(42): p. 38433-40.

14.   Mirzabekov, T., et al., *Enhanced expression, native purification, and characterization of CCR5, a principal HIV-1 coreceptor.* J Biol Chem, 1999. **274**(40): p. 28745-50.

15.   Endres, M.J., et al., *CD4-independent infection by HIV-2 is mediated by fusin/CXCR4.* Cell, 1996. **87**(4): p. 745-56.

16.   Mansfield, R., et al., *CCR5 pharmacology methodologies and associated applications.* Methods Enzymol, 2009. **460**: p. 17-55.

17.   Myszka, D.G., *Biacore- yeah it can do that!, DIPIA 2010*. 2010.