Investigating the Interaction between the Tetraspanin CD82 and Gangliosides

And

Investigating the Binding Partners of CLEC14A and the Function of the CLEC14A Extracellular Domain

By Puja Lodhia

A dissertation submitted to the University of Birmingham in partial fulfilment of the Degree of MRes Biomedical Research

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Abstract

CD82 is a tetraspanin involved in tumour metastasis suppression. It is downregulated in several cancers including breast, liver and prostate cancer. Although interactions with gangliosides and cholesterol have been demonstrated, no ligand has yet been identified to directly target CD82. CD82 interaction with gangliosides has been explored by immunoprecipitation from cell lysates. However, direct interaction of purified full length CD82 protein with ganglioside has not yet been shown. This study demonstrates the interaction of full length CD82 protein with the ganglioside GM2 by ELISA, and did not require the presence of any other components of the cell membrane. This interaction was further explored by surface plasmon resonance and solid state NMR techniques.

Acknowledgements

Many thanks to all the members of the Overduin Lab for their help over the course of this project. I would like to thank Dr R. Sundaresan for his guidance and supervision and to Dr M. Lenoir for his assistance with surface plasmon resonance experiments. Thank you also to Dr M. Overduin, Dr F. Berditchevski and Dr E. Odintsova for their input and comments.

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1. Introduction

1.1 Tetraspanins

Tetraspanins are a family of transmembrane proteins found in all eukaryotic cells and are characterised by four membrane spanning domains connected by two extracellular loops. Although there are many membrane proteins with four transmembrane domains, the tetraspanin group is distinguished by several conserved amino acid sequences including the CCG motif in the large extracellular loop (1). To date, 33 tetraspanins have been observed in human cells. Their functions range from controlling cell motility to mediating fertilisation, viral infection and immune response. Tetraspanins are thought to mediate most of their activities via lateral interactions with neighbouring components of the cell membrane. It has been observed that tetraspanins form clusters in the cell membrane known as 'tetraspanin enriched microdomains' or TERMs. These microdomains are highly populated by tetraspanins as well as other membrane proteins such as integrins and gangliosides, facilitating intermolecular interactions (1).

1.1.2 Tetraspanin Enriched microdomains

Tetraspanin enriched microdomains (fig. 1), are areas of the plasma membrane highly populated by tetraspanins. TERMs originally came to light due to their resistance to treatment with detergents. Studies revealed TERMs to be rich in several other membrane molecules including integrins, receptor tyrosine kinases and G-protein coupled receptors, giving rise to the notion that close association of these molecules favours formation of complexes with tetraspanins, resulting in signal transduction. There are three types of interaction known to occur within TEMs. (i) covalent interactions between tetraspanin and integrins and some tetraspanin-tetraspanin associations, (ii) non-covalent tetraspanin-tetraspanin interactions and (iii) non-direct interactions with cholesterol and gangliosides (1)(2). For the majority of tetraspanins, no ligands have been discovered. Hence, interest has been focused on the lateral interactions of

tetraspanins and their neighbouring membrane components to elucidate their function. In particular, the tetraspanin web and its composition is thought to be a key factor influencing tetraspanin activity by bringing molecules into close proximity and thus encouraging cross-talk between them. The TERM is highly dynamic, with molecules constantly moving into or out of the microdomain and the changes in TERM composition determine tetraspanin activity (3).

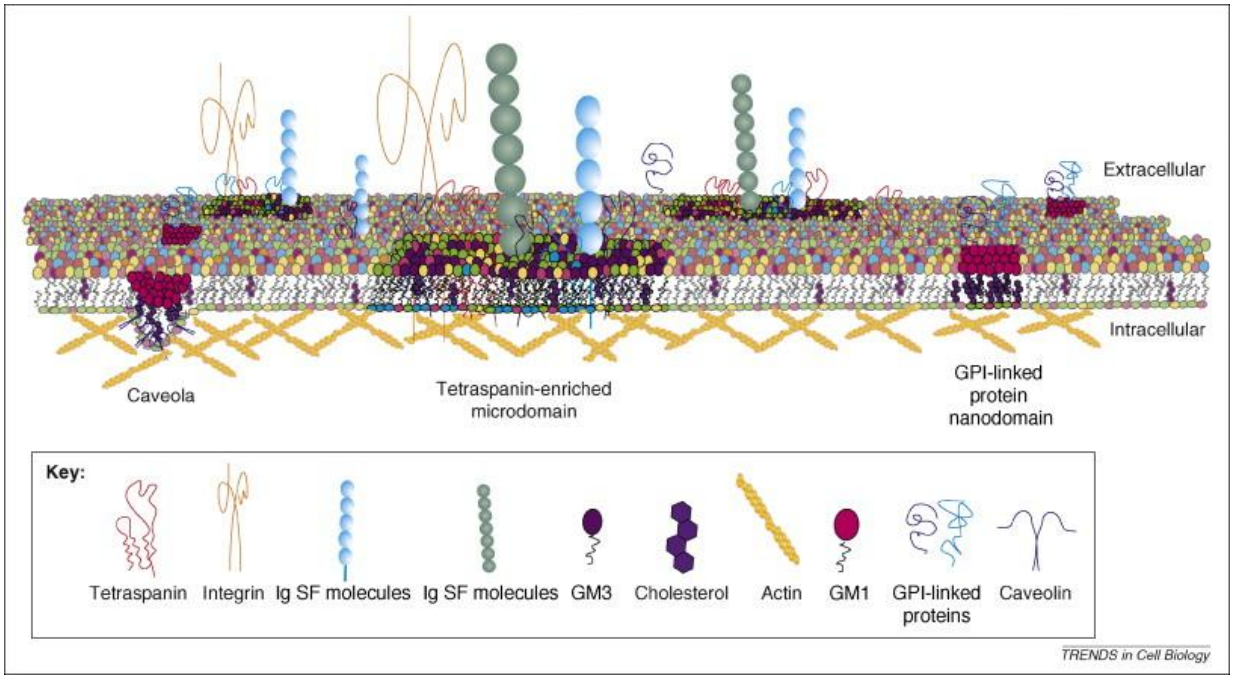

Figure 1. Structure of Tetraspanin Enriched Microdomains (TERM). Tetraspanins form clusters in the plasma membrane. TEMs are highly populated by tetraspanins as wells as associated molecule including integrins, gangliosides and Ig molecules. TEM composition regulates signalling across the membrane (3).

1.2 CD82

CD82, also known as TSPAN27 or KAI1, is a tetraspanin initially found to be involved in activation of T-cell receptors in lymphocytes. It is highly expressed in prostate, lung and liver tissues as well as in leukocytes (4). It was first implicated in cancer by a genetic screen for metastasis suppressors in prostate cancer. Orthotopic implantation of lung cancer cells infected by adenovirus encoding CD82 in a rat model showed reduced tumour metastasis to the lymph nodes (5). Breast cancer cell lines transfected with CD82 show decreased migration and invasion while CD82 knock-out cells exhibited increased motility (6). Lack of CD82 is associated with poor

prognosis in prostate and breast cancer patients (7). Inhibition of metastasis is critical for the development of highly effective cancer therapies as the major cause of death for cancer patients is not the growth of the primary tumour but metastasis to other anatomical regions. The role of CD82 in tumour metastasis is therefore of considerable interest.

1.2.1 CD82 Structure

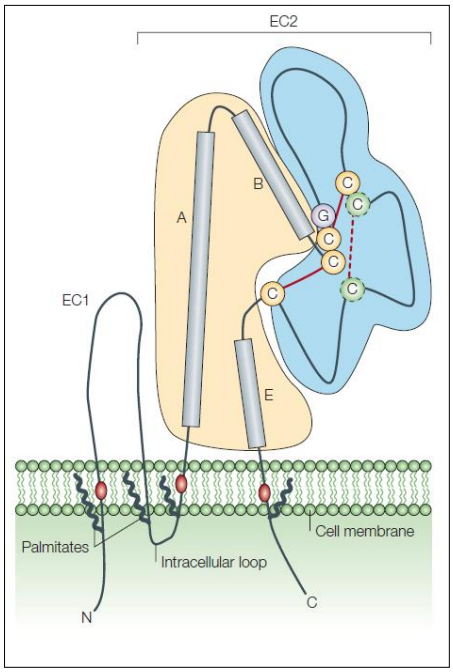

Figure 2. Structure of Tetraspanins. Tetraspanins contain four transmembrane domains linked by small and large extracellular loops (SEL and LEL), with unstructured C and N terminal tails. The LEL consists of a constant domain depicted in yellow, and a variable domain shown in blue. The constant domain contains three α-helices while the variable region contains two α-helices and a highly conserved CCG motif. Two disulphide bridges are essential for correct LEL folding. Tetraspanins are also palmitoylated at membrane proximal cysteine residues (1).

The atomic resolution structure of CD82 has not been determined, but has been modelled on the closely related tetraspanin CD81 (fig. 2)(1). CD82, like all members of the tetraspanin family, possesses four transmembrane domains linked by small and large extracellular loops (SEL and LEL) with short intracellular N and C terminal tails. The LEL comprises a large portion of the protein and consists of a constant region which is conserved in other tetraspanins, and a variable domain which differs in sequence and structure across the tetraspanin family and is thought to give rise to the specific functions of different tetraspanins despite their homology (8). The constant domain of the LEL is known to contain 3 α-helices. The variable domain of the LEL contains two α-helices (8) and is thought to be involved in interactions with other membrane components. It features a conserved CCG motif of which the two cysteine residues form

disulphide bonds with cysteines in other parts of the loop to fold the protein into its correct conformation (9). CD82 is heavily glycosylated, ranging in molecular weight from 28KDa to up to 60KDa. It is also palmitoylated at intracellular cysteine residues proximal to the plasma membrane and this post translational modification is shown essential for correct trafficking of CD82 to the membrane (10).

1.2.2 CD82 Function

Cholesterol and gangliosides have been shown to interact with CD82. However, no ligand has been determined to directly bind to CD82, and the tetraspanin does not possess any enzymatic activity (9). Its effects are therefore thought to be mediated by lateral interaction with other membrane molecules. The mechanism by which CD82 inhibits metastasis is unclear although several possibilities have come to light including interactions with integrins, other tetraspanins and receptor tyrosine kinases (fig. 3)(9).

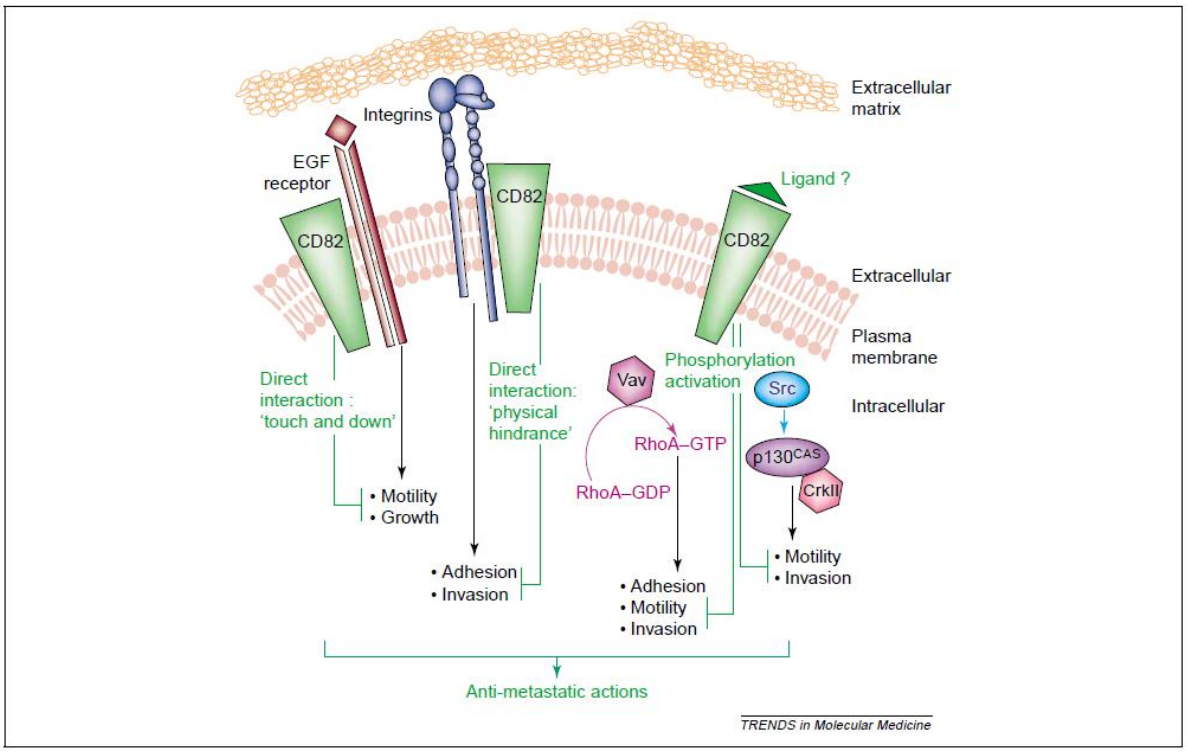

Figure 3. Anti-metastatic Actions of CD82. CD82 interacts with integrins which mediate cell adhesion and invasion while interaction with the EGF receptor regulates cell motility to inhibit tumour metastasis(9).

1.2.3 CD82 Interaction with Integrins

Integrins are transmembrane proteins which are involved in mediating cell attachment to components of the extracellular matrix (ECM) such as laminin and fibronectin (fig. 3). They have a major role in regulating cell motility but are also involved in cellular signalling. They have been found to localise to tetraspanin webs and are known to interact with tetraspanins (9). CD82 was found to co-immunoprecipitate with α4β1 integrin (11). Co-localisation of CD82 and α4β1 was also observed by confocal microscopy. Ovarian cancer cells transfected to overexpress CD82 exhibited increased adhesion via α5β3 integrin and reduced cell motility, suggesting that the anti-metastatic actions of CD82 may be mediated by interaction with integrins by regulating cell adhesion (12).

1.2.4 CD82 Interaction with Receptor Tyrosine Kinases

Interaction of CD82 with receptor tyrosine kinases such as c-met and EGFR (epithelial growth factor receptor) has been observed. EGFR is a cell surface receptor activated by growth factors and activates pathways responsible for cell proliferation, migration and DNA synthesis. Dysregulation of EGFR is therefore implicated in cancer. CD82 associates with EGFR and triggers internalisation of the receptor by endocytosis to reduce surface levels of EGFR (13). Reduced activation of EGFR resulted in decreased formation of lammelipodia and decreased cell migration, providing the basis of a possible mechanism for the anti-metastatic effect of CD82. CD82 co-immunoprecipitates with protein kinase C, calveolin-1 and the ganglioside GM3, components that are commonly found in tetraspanin webs. CD82 associates with EGFR and recruits calveolin-1. Calveolin-1 recruits protein kinase C which interacts with EGFR and promotes receptor internalisation. This data suggests formation of a complex consisting of EGFR, CD82, GM3, calveolin and protein kinase C and reveals the importance of TERMs for mediating tetraspanin activity (14).

C-met is a receptor tyrosine kinase involved in wound healing. It is frequently upregulated in prostate cancer and is associated with increased tumour metastasis and tumour related angiogenesis. CD82 is able to inhibit c-met activation in two ways. It can prevent interaction of c-met with its ligand HGF (hepatocyte growth factor) and it can also disrupt ligand independent activation of c-met by integrins. CD82 upregulation also correlates with reduced activation of signalling molecules downstream of c-met including FAK (focal adhesion kinase), the tyrosine kinase Src, and p130cas. P130cas is of particular interest as it activates the Rac pathway which mediates cytoskeletal reorganisation for cell motility. Inhibition of c-met by CD82 is therefore another possible mechanism for metastasis suppression (15). However, a direct interaction between CD82 and c-met has yet to be demonstrated. It has been suggested that these two molecules may not be directly associated, but instead rely on recruitment of adapter proteins such as Grb2 to mediate signalling between them (15).

Several studies have documented that overexpression of CD82 has an inhibitory effect on Src. Src is involved in the formation of focal adhesion complexes and is therefore implicated in tumour metastasis. CD82 upregulation reduces Src activity and increases cell aggregation while a more recent study found that in addition to inhibiting cell motility via Src, CD82 expression downregulated VHL and HIF1a downstream of Src, suggesting a possible role for CD82 in inhibiting angiogenesis, a process which is associated with metastasis (16).

In addition to integrin mediated cell adhesion, CD82 may also influence adhesion via E-cadherin and β-catenin. CD82 upregulation increased E-cadherin mediated cell adhesion. Immunoflourescent staining revealed that CD82 expression induced relocalisation of β-catenin to E-cadherin adhesion complexes, possibly stabilising the adhesion complex to reduce cell motility (17).

1.2.5 Other Activities of CD82

Most research on CD82 has focused on its anti-metastatic role and reduction in cell motility. However, metastasis is a complex process encompassing several events including breakdown of the extracellular matrix to allow cell migration, evasion of anoikis upon cell detachment, invasion of blood vessels and angiogenesis. CD82 has also been implicated in these processes.

CD82 expression impairs synthesis of urokinase plasminogen activator receptor (uPAR), an enzyme which breaks down components of the ECM, a process which occurs at early stages of metastasis. This prevents cells from disseminating from the primary tumour and inhibits tumour invasion of the surrounding tissue (18). A separate study found that CD82 stabilises the interaction between uPAR and the integrin α5β1. This association disrupts binding of uPA ligand to the receptor, inhibiting activation of proteolysis pathways (19). A screen for genes involved in apoptosis highlighted CD82, and further research found that CD82 is associated with increased production of reactive oxygen species which trigger apoptosis (20).

1.3 CD82 and Gangliosides

Gangliosides are part of the glycosphingolipid family of glycoproteins which are present in eukaryotic cell membranes. Glycosphingolipids consist of a hydrophilic carbohydrate head group which protrudes from the cell membrane and a ceramide tail embedded in the external leaflet of the bilayer (fig. 4) (21). Gangliosides, so called due to their abundance in ganglions, possess a sialic acid residue attached to the head region. The number of sialic acid residues and their position varies between different gangliosides. Gangliosides are over-expressed in a range of cancers and have been pursued as a target for tumour immunotherapy (22). Gangliosides are involved in various cellular processes including modulation of enzyme activity and cellular

signalling. They can interact with selectins to modulate cell adhesion and have also been shown to interact with growth factor receptors such as EGFR and with integrins. They are also able to form lipid domains within the cell membrane and are often present in the tetraspanin web (23).

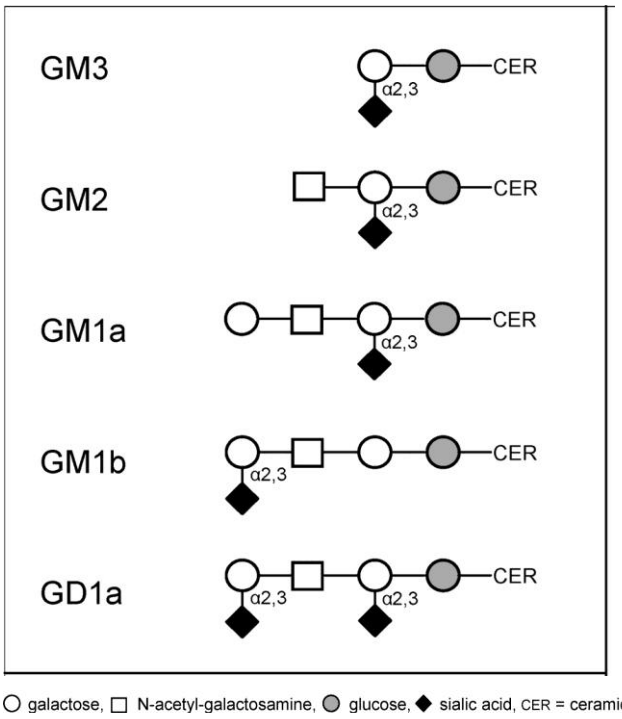

Figure 4. The Structure of Gangliosides. Gangliosides consist of a hydrophilic head group and a hydrophobic ceramide tail. The head group contains glucose and galactose as well as sialic acid residues. The number and position of sialic acid residues varies between gangliosides (24).

The interaction of gangliosides with tetraspanins is under investigation. It has been observed that levels of gangliosides GM1 and GD1a were significantly increased in HB2 cells transfected to over-express CD82 compared to control HB2 cells. Immunofluorescence revealed that GD1a co-localised with clusters of CD82 and EGFR on the cell membrane. Other studies have noted that GD1a expression has an inhibitory effect on EGFR signalling suggesting that GD1a may be involved in regulating the interaction between CD82 and EGFR (25). Inhibition of ganglioside synthesis resulted in reduced co-immunoprecipitation of CD82 with known binding partners including EGFR, α3 integrin and CD151, supporting the notion that gangliosides are involved in stabilising interactions of CD82 (26). Ganglioside depletion resulted in the redistribution of CD82 from the cell periphery to random clusters over the cell surface suggesting a role for gangliosides in organising the distribution of tetraspanins in TEMs. Ganglioside depletion in HB2/CD82 cells

resulted in reduced EGFR phosphorylation while control HB2/Zeo cells showed no change in EGFR phosphorylation, suggesting that CD82 is required for gangliosides to modulate EGFR activity.

The interaction between CD82 and GM2 was demonstrated by using a pull down assay from YTS1 bladder cancer cells transfected to express CD82. CD82 was found to bind to GM2 coated beads while beads coated with GM3 or Gb4 did not show any CD82 binding suggesting that CD82 interacts with GM2 directly and specifically. CD82 and GM2 were also observed to colocalise by confocal microscopy. Inhibition of ganglioside synthesis resulted in increased motility of YTS1/CD82 cells in response to HGF, while YTS1 control cells showed no difference (27). A separate study by the same group using silica nanospheres coated with gangliosides revealed that nanospheres coated with a complex of GM2 and GM3 had a significantly greater inhibitory effect on motility of YTS1 cells than either ganglioside alone. CD82 also bound to a greater extent to beads coated with the GM2/GM3 complex suggesting that the GM2/GM3 heterodimer may interact with CD82. Cells treated with nanospheres coated with the heterodimer showed reduced activation of the cMet pathway which has been implicated in CD82 mediated metastasis suppression. This suggests that the GM2/GM3 heterodimer interacts with CD82 and modulates its effects on the cMet pathway (28).

1.4 Aims

Interaction of full length CD82 protein expressed in mammalian cells with the ganglioside GM2 has previously been demonstrated by co-immunoprecipitation using cellular lysates. The aim of this study is to determine if a direct interaction between CD82 and GM2 or GD1a occurs using purified full length CD82 protein produced in yeast. The CD82-ganglioside interaction will be assessed by several techniques including ELISA, surface plasmon resonance and solid state NMR in order to better understand the role of the CD82-ganglioside interaction in the inhibition of cancer metastasis.

2. Materials and Methods

2.1 Antibodies and Reagents

The anti-CD82 monoclonal antibody M104 and anti-CD81 M38 antibody were kindly provided by Dr O. Yoshie (Shionogi Institute, Osaka, Japan). The anti-CD82 monoclonal antibody TS82 was kindly provided by Dr E. Rubinstein (INSERM U268 Villejuif, France). Anti-CD82 monoclonal antibody ɣC12 was kindly provided by Dr H. Conjeaud (Institute Cochin, Paris, France). Anti-CD82 antibody TS82b was purchased from Abcam Plc. Anti-His-HRP conjugated antibody was purchased from Invitrogen. Polyclonal goat anti-mouse and polyclonal goat anti-rabbit HRP conjugated antibodies purchased from Dako were used as secondary antibodies for Western Blotting. Anti-GM2 polyclonal antibody was purchased from Abcam Plc. Additional anti-GM2 polyclonal antibody was purchased from Calbiochem. Monosialoganglioside GM2 and disialoganglioside GD1a were purchased from Matreya LLC Lipids and Biochemicals.

2.2 Production of Full Length CD82 Protein

Full length CD82 protein was expressed in Pichia Pastoris as described in Jamshad et al, 2008 (29) by R. Sundaresan. The cell pellet was resuspended in 50 mM Tris pH 8.0, 200 mM NaCl, 0.5 mM EDTA, 2mM TCEP and Complete protease inhibitor (Roche). Cells were lysed by pressure cell disruption at 30,000psi. Cell debris was removed by centrifugation at 10,000g for 30 minutes. The supernatant was ultracentrifuged at 100,000g for 1.5 hours. The supernatant was removed and DPC (dodecylphophocholine) or β-OG (β-octyl glucoside) was added to the protein pellet to a final concentration of 2%. The protein was purified by filtration through a nickel sepharose column. Three glycosylation sites in the extracellular domain were mutated from Asn to Gly and five palmitoylation sites in the membrane proximal region were mutated from Cys to Ala to obtain homogenous protein samples.

2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) and Western Blotting

All gels were run using Biorad Pre-Cast 18 well gels. Samples were loaded with Laemmli Buffer containing bromophenol blue and if required, were reduced with 99% β-mercaptoethanol (Sigma Aldrich). Gels were stained with Instant Blue Coomassie Stain (Expedeon) or used for Western Blotting. SDS PAGE separated proteins were transferred to a nitrocellulose blotting membrane (Biotrace, Life Sciences). Membranes were blocked with 5% skimmed milk-PBST for one hour, and then probed with the appropriate primary antibody for one hour. Membranes were washed 3x20 minutes with PBST and incubated in secondary antibody for one hour. Blots were developed using Western Lightning Plus Chemiluminescence Kit (Perkin Elmer Inc.) and detected by x-ray film (Amersham Hyperfilm MP, GE Healthcare Ltd.).

2.4 Immunoprecipitation

Protein G Sepharose beads (Amersham Biosciences) were washed with 1% Triton PBS buffer. Beads were incubated with TS82, TS82b, ɣC12, M104 or M38 antibody overnight on a rotary mixer at 4°C. Beads were pelleted and washed. Beads were then incubated with 1 µL of CD82 for 1 hour at room temperature on a rotary mixer. Beads were washed with 1% Triton PBS buffer, resuspended in Laemmli buffer and heated to 95^{o}C for 5 minutes to elute protein. Samples were then run on SDS gels. Western blots were carried out using TS82b antibody.

2.5 ELISA

Plates were incubated overnight with 10 µM GM2 or GD1a diluted to 10 µM in 9:1 methanol and water. Plates were washed with PBS 0.001% Tween and blocked with 3% BSA PBST. Plates were washed and CD82 diluted in PBS 0.001% Tween was added to the relevant wells and incubated for 1 hour. Plates were washed and incubated with TS82 antibody (1:1000 dilution in PBS 0.001% Tween) for 1 hour, followed by incubation with mouse secondary antibody (1:10000

diluted in 3%BSA). Presence of GM2 or GD1a was confirmed by incubating wells with GM2 or GD1a antibody followed by the relevant secondary antibody. Plates were developed with TMB Reagent (Pierce) and absorbance was detected at 630nm with an Elx800 plate reader.

2.6 SPR

Sensor chips CM5, L1 and NiNTA were purchased from Biacore, GE Healthcare. HBS buffer, PBS buffer, amine coupling kits and nickel coating kits were also purchased from Biacore, GE Healthcare. SPR was conducted with a Biacore 3000 instrument. Ligands were immobilised to the chip and interaction with the analyte was detected as a change in angle of resonance represented as response units. NiNTA chips were prepared for ligand binding using a nickel coating kit as per manufacturer's instructions. Where required, ligands were amine coupled to the L1 or CM5 chips using an amine coupling kit as per manufacturer's instructions. Several SPR approaches were used. 1. An L1 chip was coated with POPC *(*Palmitoyl-oleoyl-phosphatidyl-choline) and POPC ganglioside liposomes and binding was detected by anti-ganglioside antibody. 2. NiNTA chip was coated with CD82 (0.25%DPC) and binding was detected by anti-HIS antibody. 3. CM5 chip was coated with anti-HIS antibody to capture CD82 protein. 4. HIS antibody was amine coupled to CM5 chip to capture CD82. 5. GM2 was amine coupled to CM5 chip and binding was detected with anti-CD82 antibody. 6. GM2 was amine coupled to L1 chip. CD82 was passed over the flow cells and binding detected by anti-HIS or TS82 antibody. Gangliosides and CD82 were diluted to 10 µM in the relevant flow buffer. Antibodies were diluted to 1:500 in the relevant flow buffer. Chips were washed with 100 mM NaOH and 40 mM CHAPS. 3% BSA was used as a blocking buffer.

2.7 Liposome Preparation

Liposomes were prepared with 0.5 mM POPC and 10 µM ganglioside. Solutions were dried to a film with nitrogen gas. 1 ml PBS buffer was added to each sample. Liposomes were freeze-thawed 10 times with dry ice and then centrifuged for 1 minute at 1000rpm. Liposomes

were then passed through an extruder (Avestin) with a 0.1 μm Nuclepore membrane 20 times. Liposomes were collected in an eppendorf and stored at 4C.

2.8 Sample preparation for NMR

Preliminary samples were prepared containing POPC (Avanti Polar Lipids Inc.) and POPC with GM2. 15mg POPC was added to one glass tube. 15mg POPC and 0.5μl 1mM GM2 was added to a separate glass tube. Each tube was dried with nitrogen gas and samples were resuspended in 1ml TRIS buffer (20mM, pH7.5).Samples were vortexed and left at room temperature for 1 hour. Samples were ultracentrifuged at 50000rpm for 1 hour at 4 degrees. The supernatant was removed. 1 ml TRIS buffer was added to each tube and the pellet resuspended. The samples were ultracentrifuged again at 50000rpm for 1 hour at 4 degrees. The supernatant was removed and the pellets were stored at 4 degrees prior to measurement at the University of Nottingham for solid-state NMR (ssNMR) analysis by Dr Boyan B. Bonev. ssNMR experiments were carried out on a Varian 400MHz VNMRS Direct Drive spectrometer equipped with 4 mm T3 MAS NMR probe (Varian, Palo Alto CA, USA), as described in Sanghera et al 2011.

3. Results

3.1 Immunoprecipitation

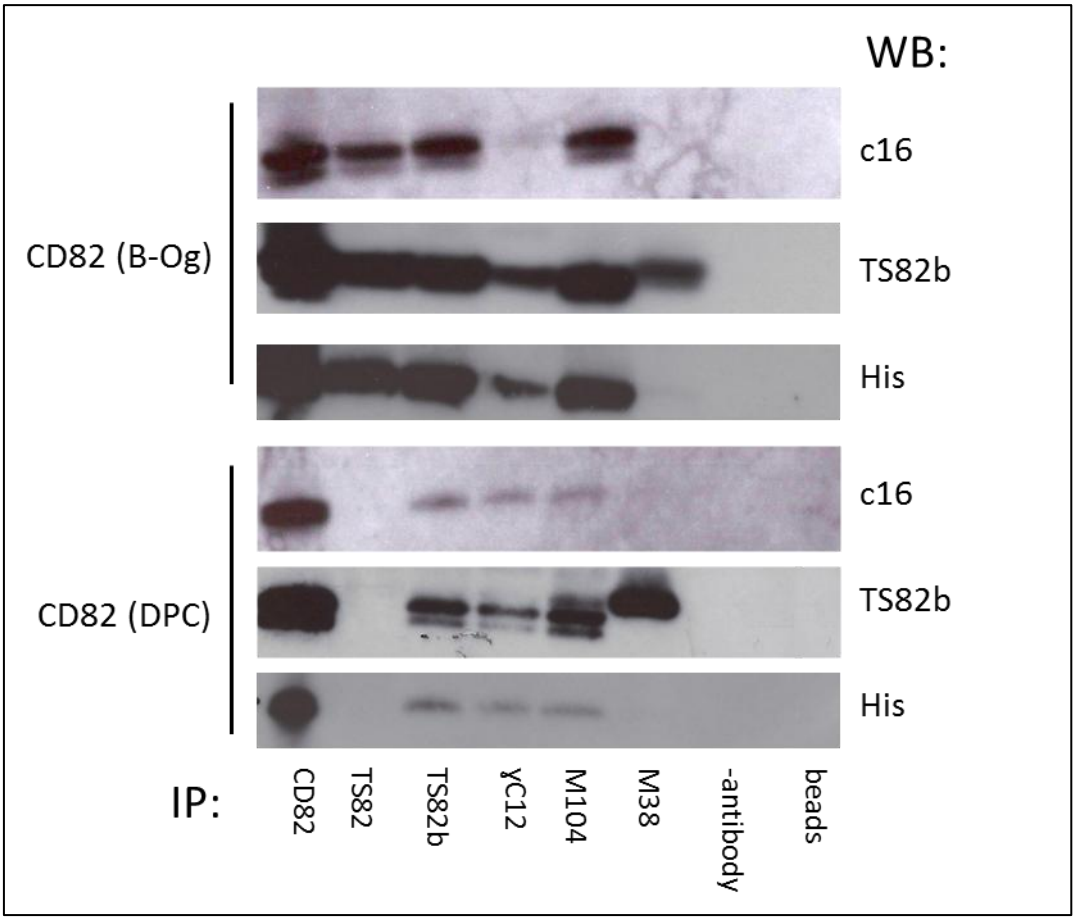

Figure 6. Immunoprecipitation of CD82 solubilised in β-OG and DPC. IgG beads were coated with TS82, TS82b, γC12 and M104 for pull down of CD82. Western blots were probed with c16, TS82b and anti-His antibodies. CD82 (β-OG) was immunoprecipitated by all anti-CD82 antibodies used. CD82 (DPC) was not immunoprecipitated by TS82 but was immunoprecipitated by other antibodies.

Immunoprecipitation of CD82 was carried out to determine the binding ability of αCD82 antibodies prior to use in ELSIA experiments. CD82 solubilised in β-OG (β-octyl glucoside) and DPC (dodecylphosphocholine) was successfully pulled down with TS82, TS82b, γC12 and M104 antibodies (fig. 6). Of these, M104 showed the strongest binding to CD82, while γC12 was the weakest. Each antibody recognises a different epitope on the CD82 protein, suggesting that protein in both samples is in the correct conformation. The TS82 antibody did not

immunoprecipitate CD82 solubilised in DPC, suggesting that binding of DPC molecules to CD82 protein may have masked the epitope for TS82. Control lanes were negative although a band was detected with the anti-CD81 antibody M38 with both β-OG and DPC solubilised proteins. These bands were only observed when blots were probed with TS82b antibody and were positioned slightly higher than the other bands for CD82. They may be due to non-specific reactivity of the M38 antibody.

3.2 ELISA Assays

Initial ELISA assays were carried out to confirm the binding of gangliosides GD1a and GM2 to the 96 well plate (fig. 7). GD1a coated wells gave a highly positive result with both antibody dilutions suggesting GD1a had bound to the plate. However, the control wells containing no ganglioside and wells without primary antibody also gave very high absorbance readings, suggesting non-specific binding of the secondary antibody. The GM2 coated wells gave absorbance values that were very similar to the un-coated control wells, also suggesting non-specific antibody activity.

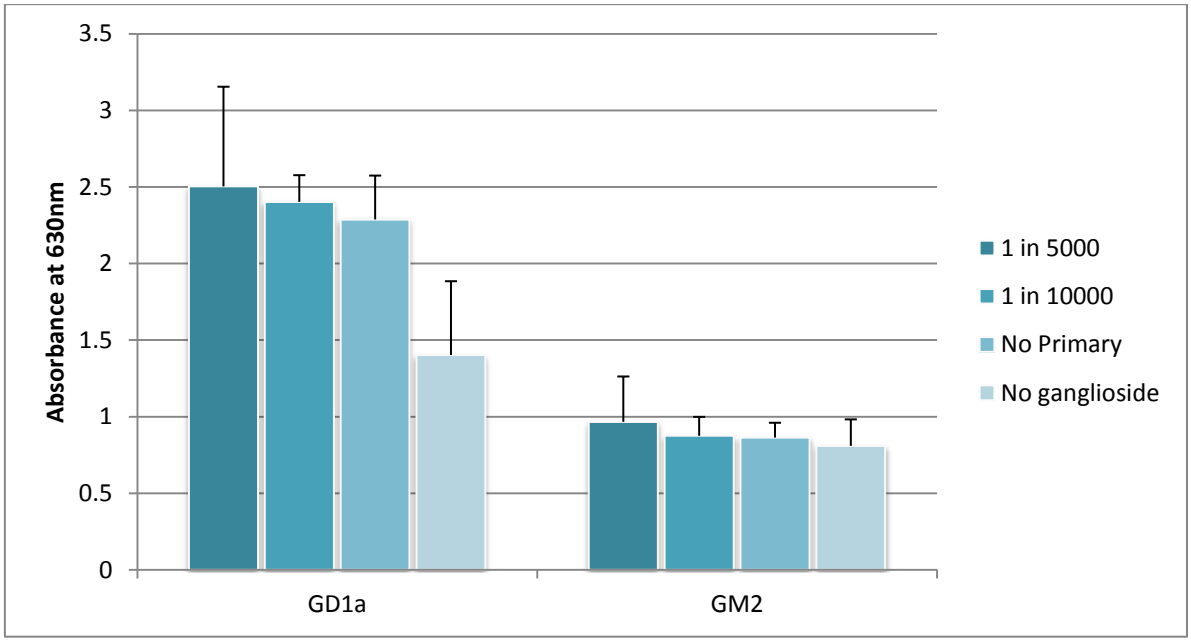

Figure 7. Titration of anti-ganglioside antibodies. GD1a and GM2 binding to the plate was detected by the relevant antibody at dilutions of 1 in 5000 and 1 in 10000 Non-specific antibody binding was detected for both GD1a and GM2. Error bars represent standard deviation.

The ELISA was repeated with decreasing concentrations of secondary antibody, and also with secondary antibody diluted in 3% BSA rather than wash buffer as indicated in the original protocol (fig. 8). Decreasing the secondary antibody concentration reduced the non-specific binding observed in the ganglioside free wells but also reduced absorbance in the ganglioside coated wells. In contrast, wells probed with secondary antibodies diluted in 3% BSA however, showed very low non-specific binding in the negative wells. A strong positive result in the GD1a coated wells confirmed that GD1a could bind to the plate. With GM2 (fig. 9), a convincing positive result was not observed in any of these conditions as the control wells and GM2 coated wells gave similar results consistently. The low activity of the anti-GM2 antibody was therefore suspected and a different GM2 antibody was ordered (Calbiochem).

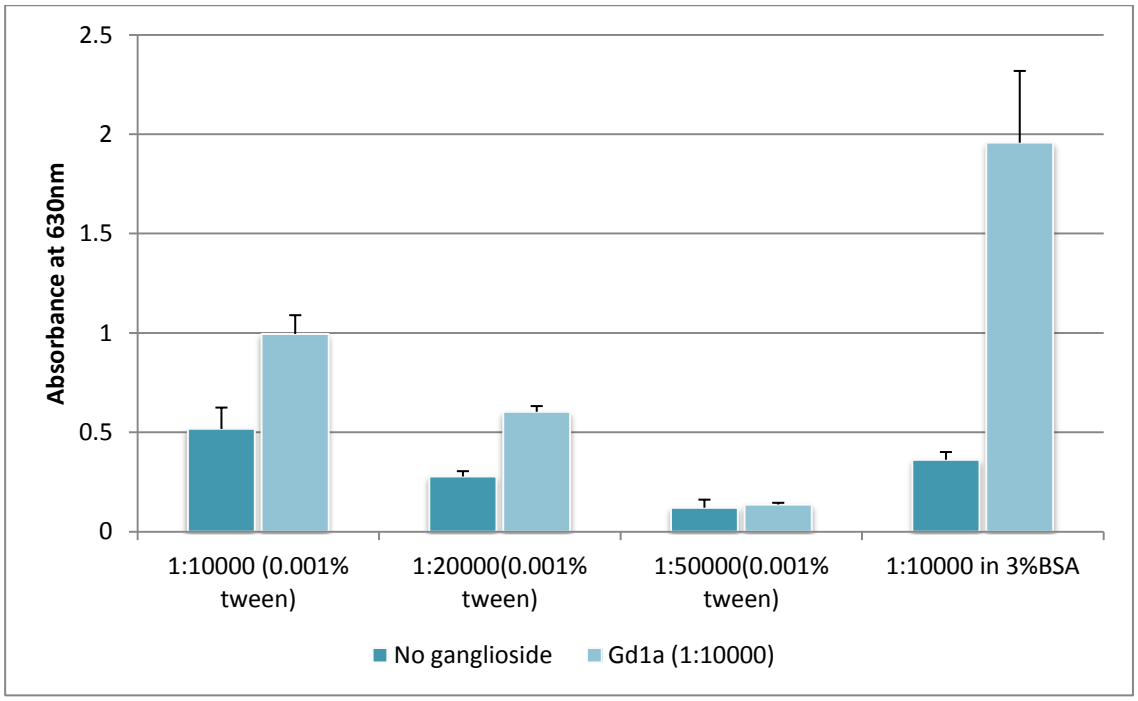

Figure 8. Detection of GD1a binding to ELISA plate using various secondary antibody conditions. Secondary antibodies diluted to 1 in 10000 in 3% BSA PBST gave the optimum result.

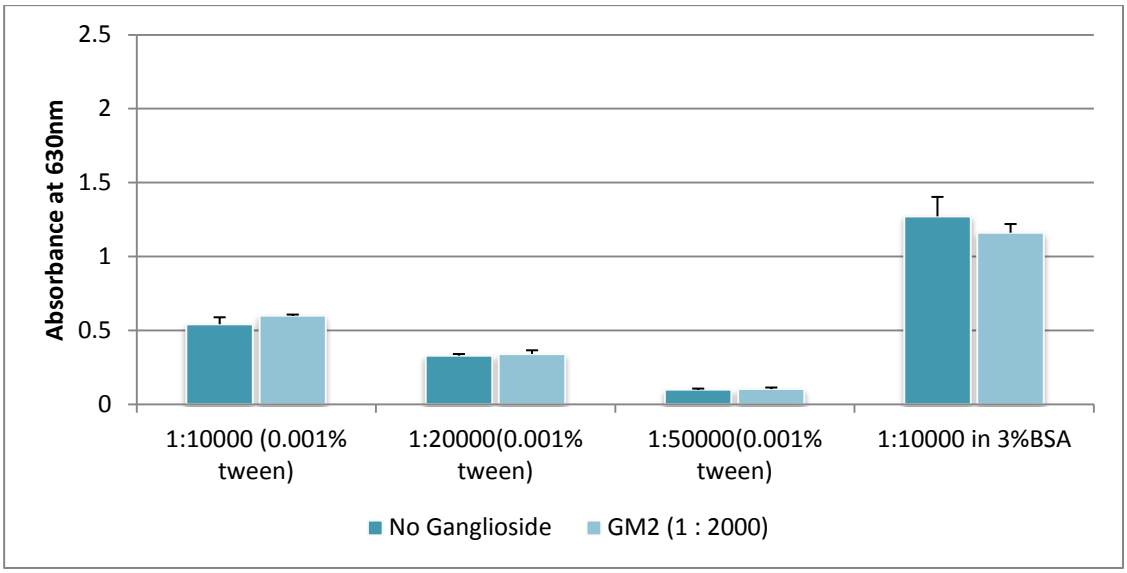

Figure 9. Detection of GM2 binding to ELISA plate using various secondary antibody conditions. High non-specific binding was observed in each case.

Activity of the new GM2 antibody was tested by ELISA (fig. 10). The antibody gave a strong positive signal at a dilution of 1:500 with wells coated with 10uM GM2. However, uncoated wells also gave an absorbance reading of 0.55 indicating non-specific binding to plates. The difference between the absorbance values in the positive and negative wells was statistically significant according to the student's unpaired T-test ($p<0.05$) and was therefore used in further experiments.

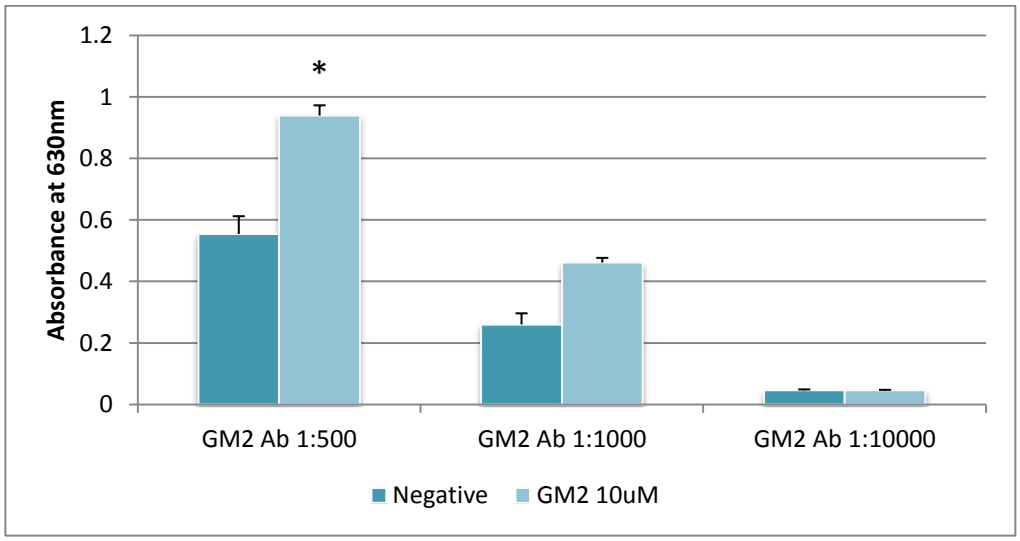

Figure 10. Testing of GM2 antibody. The second GM2 antibody detected GM2 bound to the ELISA plate when diluted to 1:500. Non-specific binding was observed in uncoated wells but the difference was statistically significant according to the student's T-test, *($p<0.05$).

A preliminary ELISA incubating ganglioside coated plates with various concentrations of CD82 (β-OG) was carried out (fig. 11). Binding of CD82 was detected using three different antibodies, TS82, M104 and HRP conjugated anti-His antibody. GM2 coated wells incubated with 50 and 100 µM CD82 gave high absorbance results, 0.6 and 0.8 respectively with TS82, suggesting that CD82 is able to bind to GM2. With GD1a, increasing concentrations of CD82 gave increasing absorbance results with both TS82 and M104 although the maximum absorbance detected was less than 0.4, suggesting that CD82 is less able to bind to GD1a than GM2 or that the GD1a-CD82 interaction is too weak to be observed by this method. Anti-His HRP conjugated antibody gave low absorbance values in each case, which in light of the positive results with both TS82 and M104 suggests that this antibody is unable to bind to CD82 in this assay.

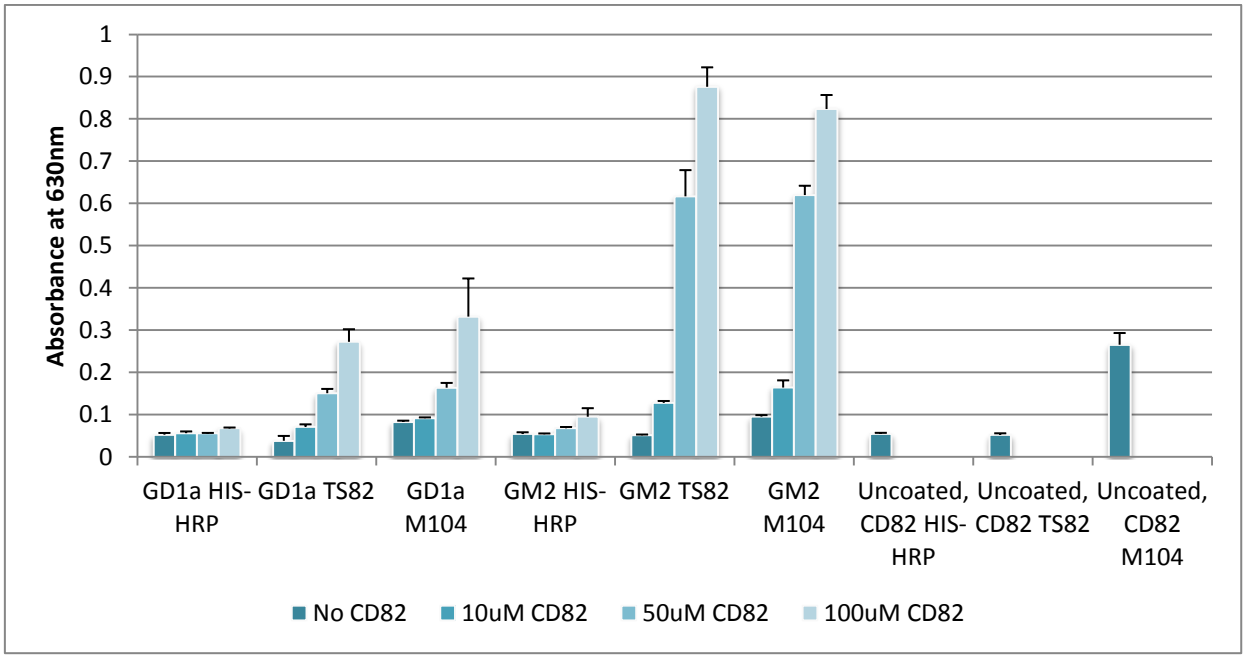

Figure 11. Preliminary ELISA with B-Og solubilised full length protein added to ganglioside coated plates. Increased absorbance was detected with GM2 coated wells incubated with 50 and 100uM CD82.

The ELISA was repeated with added control wells which were not coated with ganglioside but were incubated with CD82 (fig. 12). The wells lacking ganglioside gave absorbance readings comparable to wells coated with GD1a, with increasing concentrations of CD82 giving higher readings. These results indicated the non-specific binding of CD82 to the plate and not to GD1a.

With GM2, the absorbance readings were still significantly higher than wells containing no ganglioside ($p<0.05$), as determined by the unpaired T-test, indicating that CD82 is able to bind to GM2.

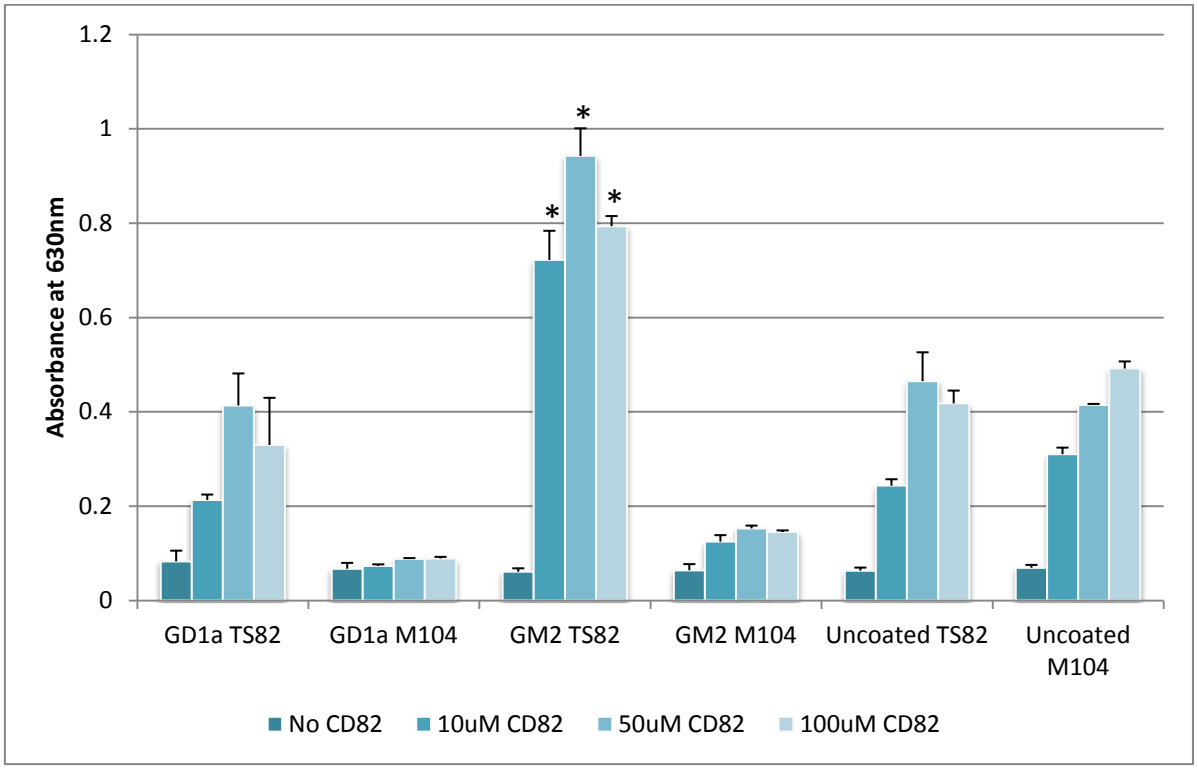

Figure 12. ELISA results of GD1a and GM2 coated plates incubated with 10, 50 and 100µM β-Og solubilised full length CD82. GM2 coated wells showed increased absorbance when incubated with CD82. The increase in absorbance was statistically significant ($p<0.05$) despite high readings for ganglioside free wells. GD1a coated wells did not show any statistically significant difference from control wells. *, results of statistical significance according to the student's T-test.

Additional ELISA assays were carried out with the aim of reducing the non-specific binding of CD82 to the plate (fig. 13). PBST, 1% BSA in PBST and 3% BSA in PBST were tested in the blocking buffers to decrease non-specific binding of CD82, by blocking any open binding sites on the plate, prior to addition of CD82.The best result was obtained with 3% BSA for ganglioside free wells. However, the absorbance was higher than with GM2 coated wells. GM2 free wells were also coated with cholesterol overnight. These wells showed reduced absorbance and therefore reduced binding of CD82 compared to GM2 coated wells although absorbance values were still high.

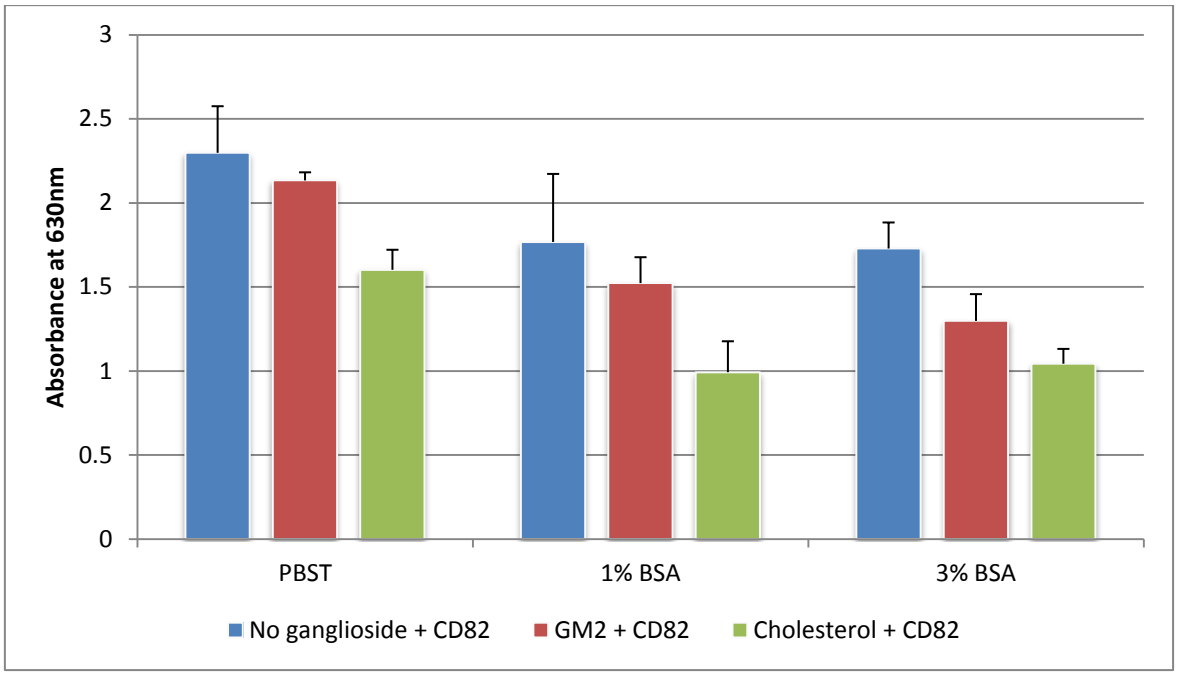

Figure 13. Optimisation of ELISA using different blocking buffers. PBST, 1% BSA, 3% BSA were tested to reduce non-specific binding of CD82. Coating the wells with cholesterol was also tested. Absorbance readings were still high in ganglioside free wells.

Additional ELISAs were carried out using shingosine and sphingomyelin to coat ganglioside free wells. Although CD82 showed reduced binding to these wells compared to GM2 coated wells, the absorbance was still higher than with uncoated wells (fig. 14).

As the β-OG solubilised CD82 protein showed high nonspecific binding in several ELISAs, assays were next carried out using the DPC solubilised protein (fig. 15). However, absorbance results for M104, anti-His antibody and TS82b antibody were all below 0.25, demonstrating that DPC solubilised CD82 did not bind to GM2 or bind non-specifically to the plate.

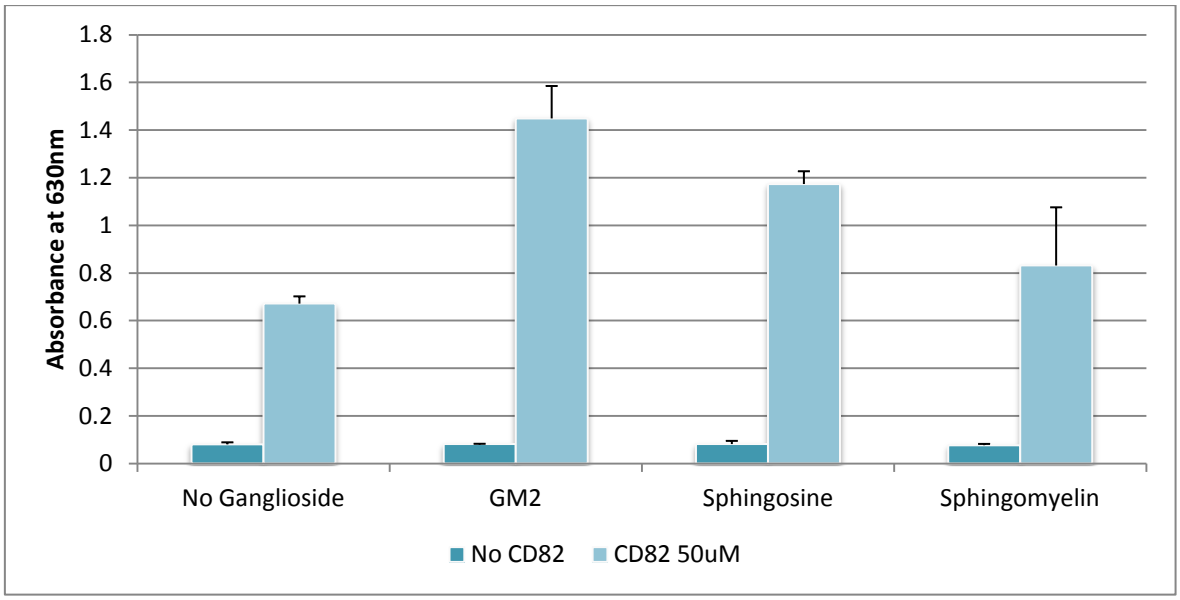

Figure 14. Blocking of uncoated wells with sphingosine and sphingomyelin. Sphingosine and sphingomyelin did not reduce non-specific binding of CD82 to the plate.

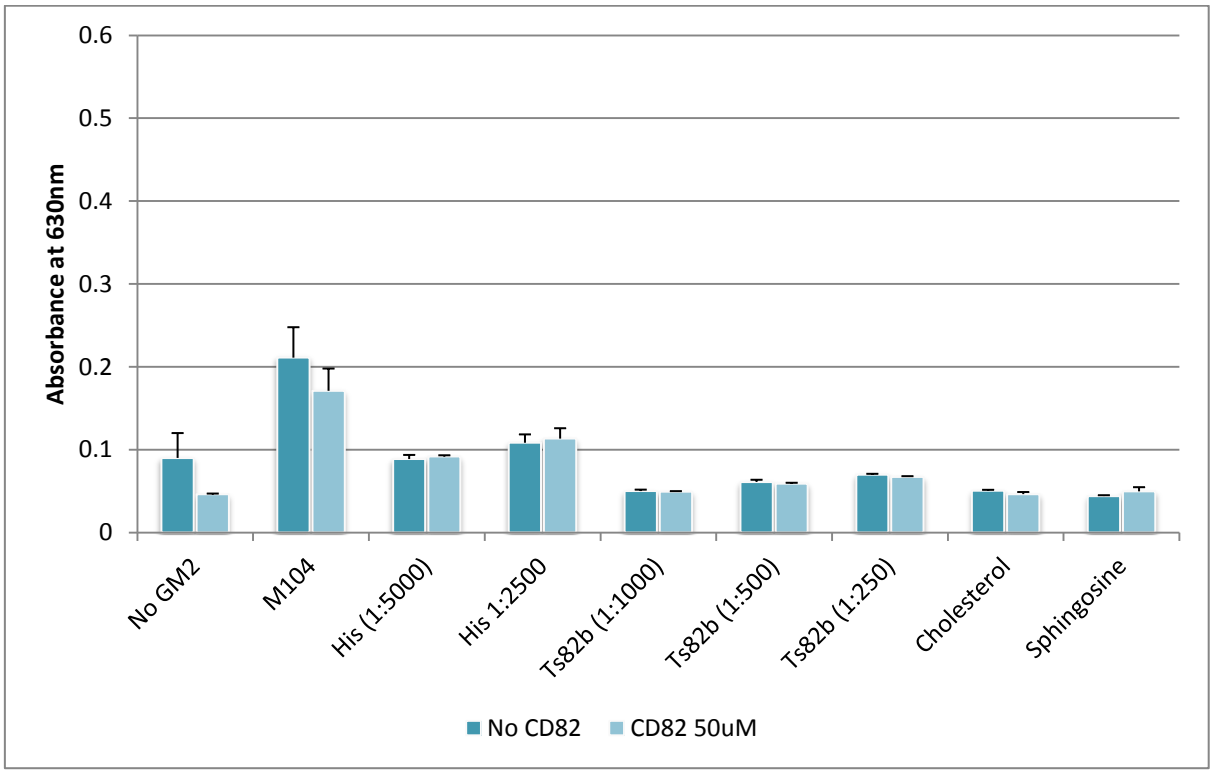

Figure 15. ELISA using DPC solubilised full length CD82 protein. Binding of CD82 to GM2 was not detected.

In an alternative approach, plates were incubated overnight with β-OG solubilised CD82 (figs. 16 and 17). Wells were incubated with 20uM GM2 for 1 hour and binding of GM2 was detected with GM2 antibody. Binding of CD82 to the plate was confirmed using TS82 antibody. Absorbance results were all below 0.3 suggesting that GM2 was unable to bind to CD82 in this assay.

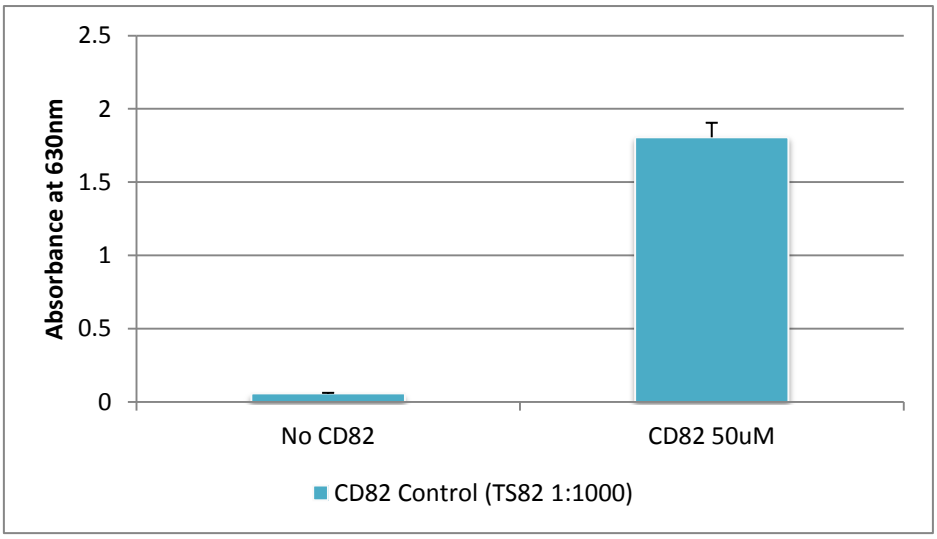

Figure 16. Confirmation of binding of β-Og solubilised CD82 to ELISA plate. Binding of CD82 to the plate was detected by TS82 antibody (1:1000)

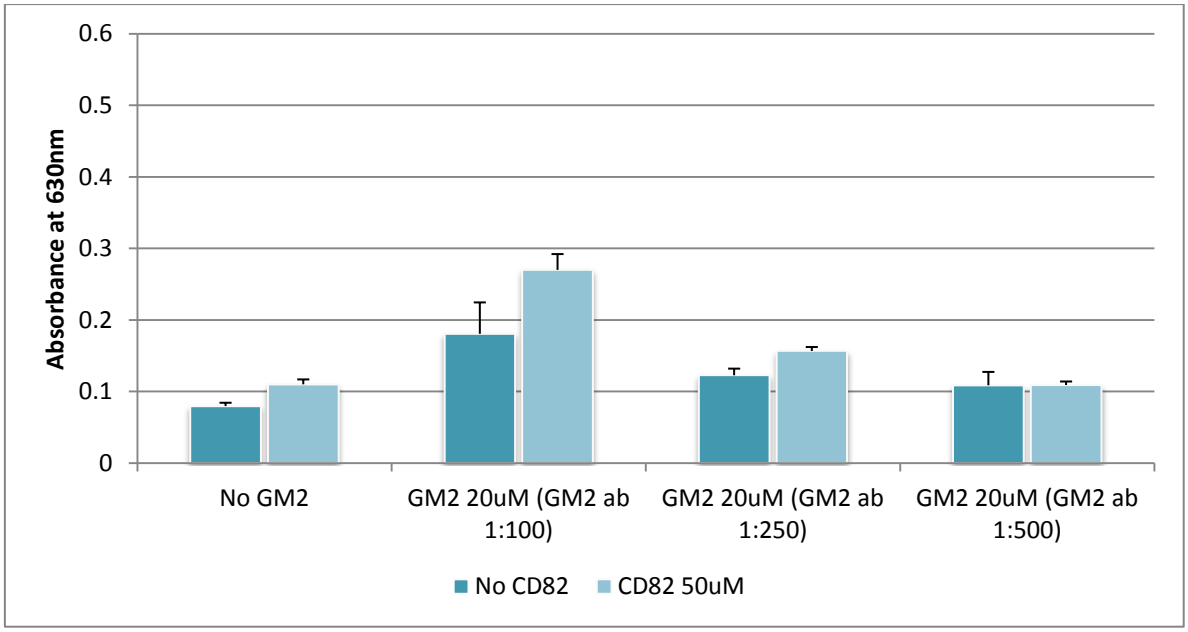

Figure 17. ELISA with wells coated with 50uM β-Og solubilised CD82. Absorbance readings were low for various concentrations of anti-GM2 antibody, indicating binding of GM2 was not detected.

3.3 Surface Plasmon Resonance

An L1 Biacore sensor chip was coated with POPC or POPC-ganglioside liposomes. Coating of the L1 chip with POPC, POPC-GD1a and POPC-GM2 liposomes was confirmed by addition of

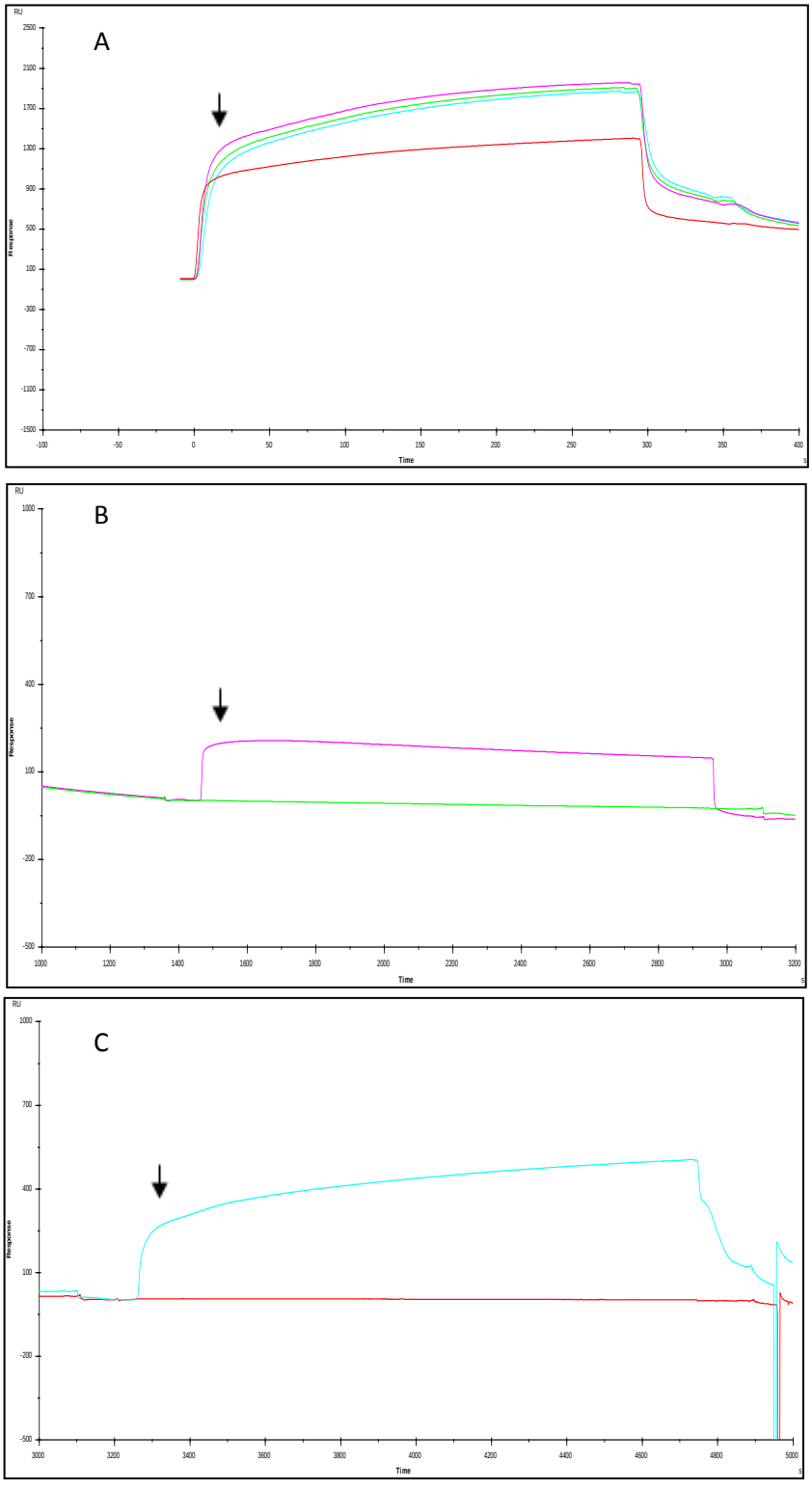

Figure 18. SPR sensograms of L1 Chip coated with POPC, POPC-GD1a and POPC GM2 liposomes. A. Coating of L1 chip with liposomes. Green, POPC, Magenta, POPC-GD1a. Cyan, POPC-GM2. Red, uncoated. Increase in relative response indicates liposome coating. ↓, addition of liposomes. B. Addition of GD1a antibody to POPC only and POPC-GD1a flow cells. Binding of GD1a antibody confirms presence of GD1a on the chip surface (magenta) while POPC only flow cell gave no response ↓, addition of GD1a antibody. C. Addition of GM2 antibody to untreated (red) and POPC-GM2 coated flow cells. Increase in relative response confirmed presence of GM2 on chip surface. ↓, addition of GM2 antibody.

anti-GD1a and anti-GM2 antibody (fig. 18). However, addition of 0.25% DPC buffer caused a sharp decrease in response, indicating removal of the liposomes coating the chip (fig. 19). The sensitivity of the liposome coating to detergent prevented addition of detergent solubilised CD82 protein to this setup.

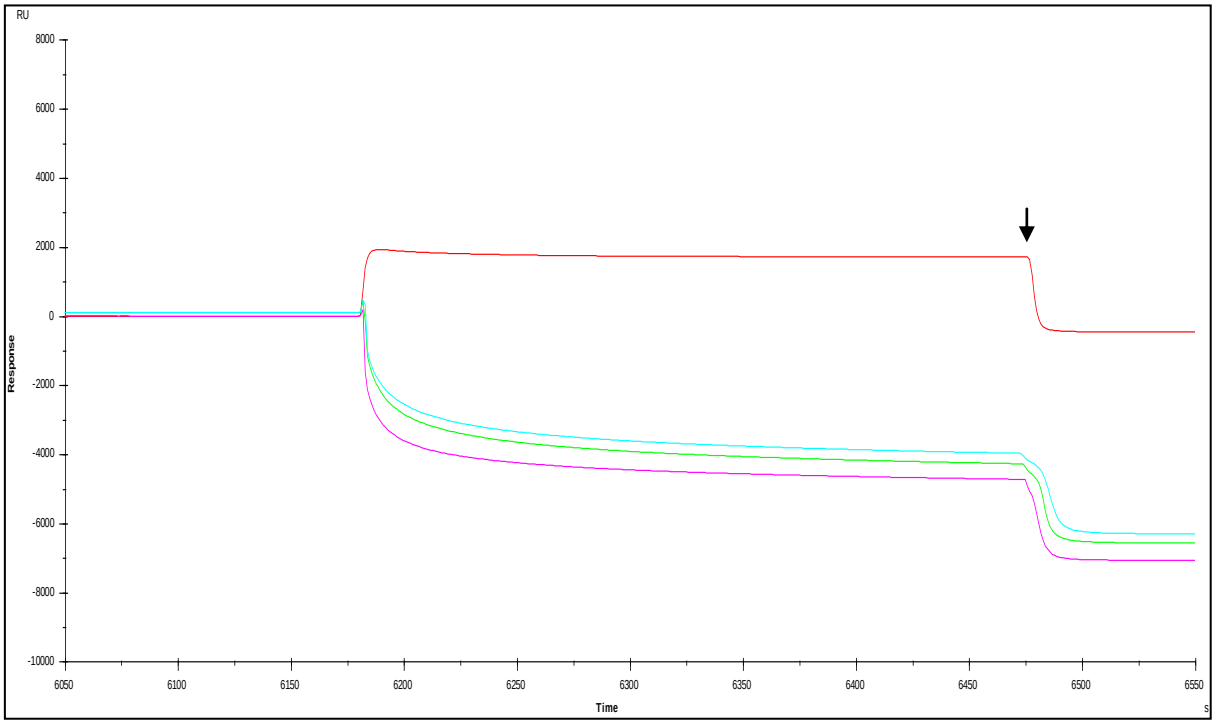

Figure 19. Addition of 0.25% DPC to the POPC, POPC-GD1a and POPC GM2 liposome coated L1 chip. ↓, addition of 0.25% DPC caused uncoating of the liposomes. Green, POPC, Magenta, POPC-GD1a. Cyan, POPC-GM2. Red, uncoated.

In an alternative approach, CD82 in 0.5% DPC was immobilised on a NiNTA sensor chip by binding of the 6xHis-tag to Ni^{2+} on the sensor chip surface (fig. 20). However, binding of CD82 was also detected in the non-nickel coated flow cell indicating non-specific protein binding. Addition of ts82b antibody gave an increase in response units for the nickel coated flow cell, but not for the non-coated flow cell, indicating binding of CD82, although the response was very low.

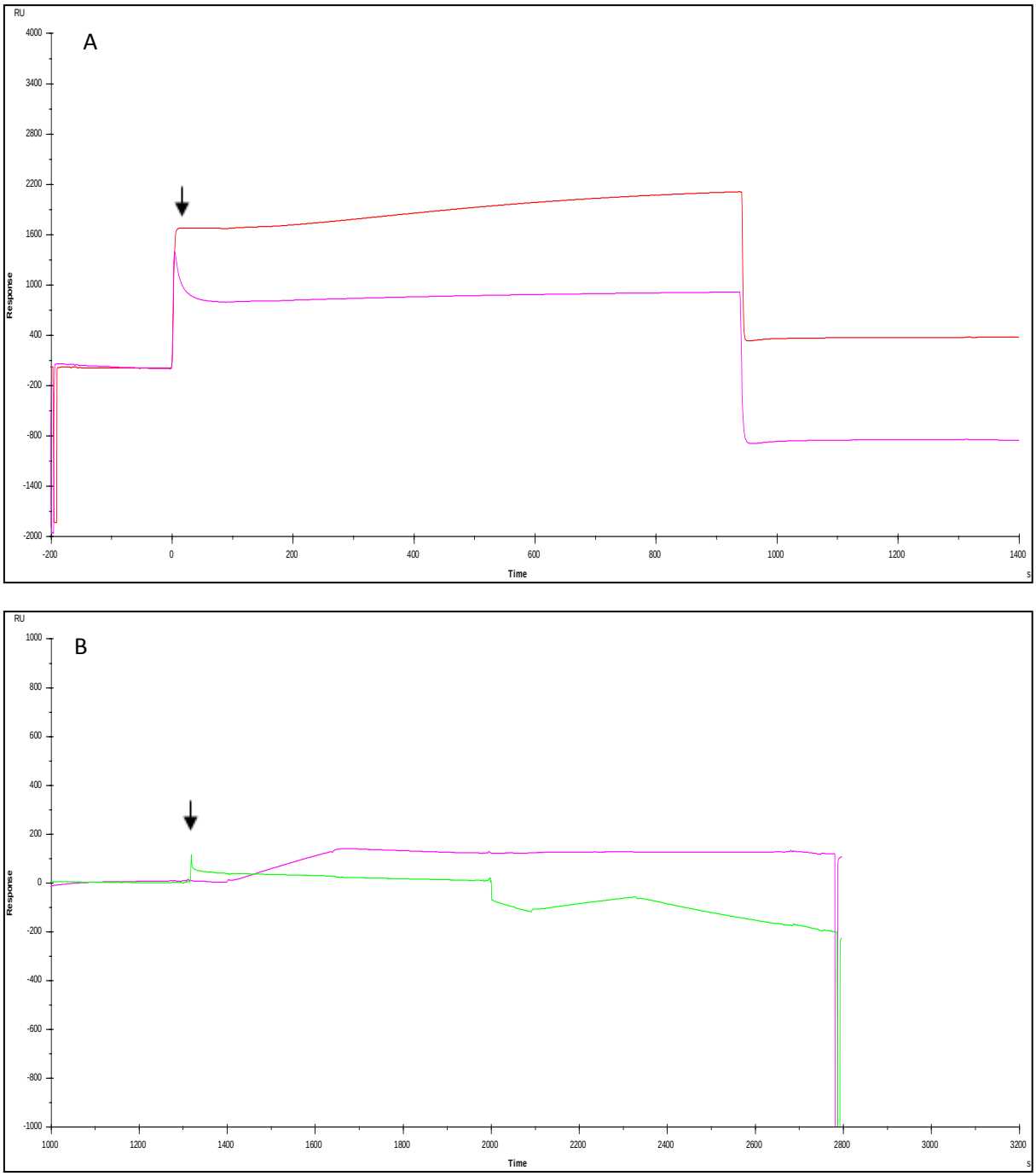

Figure 20. SPR sensograms for NiNTA chip coated with CD82. A. Coating of sensor chip surface with CD82. Magenta, nickel coated flow cell. Red, uncoated flow cell. ↓, increase in response units for the uncoated flow cell upon addition of CD82 (0.5% DPC) demonstrates non-specific interaction of CD82 with sensor chip surface. B. ↓, addition of TS82b antibody. Magenta, nickel coated flow cell loaded with CD82. Green, nickel coated flow cell without CD82. A small response to TS82b is observed in the CD82 coated flow cell.

The experiment was repeated using CD82 solubilised in 0.5% β-OG to reduce non-specific binding. Once again, CD82 interacted with both nickel coated and uncoated flow cells, indicating non-specific binding (fig. 21). Binding of CD82 to ganglioside could not be assayed by this method since proper controls could not be achieved.

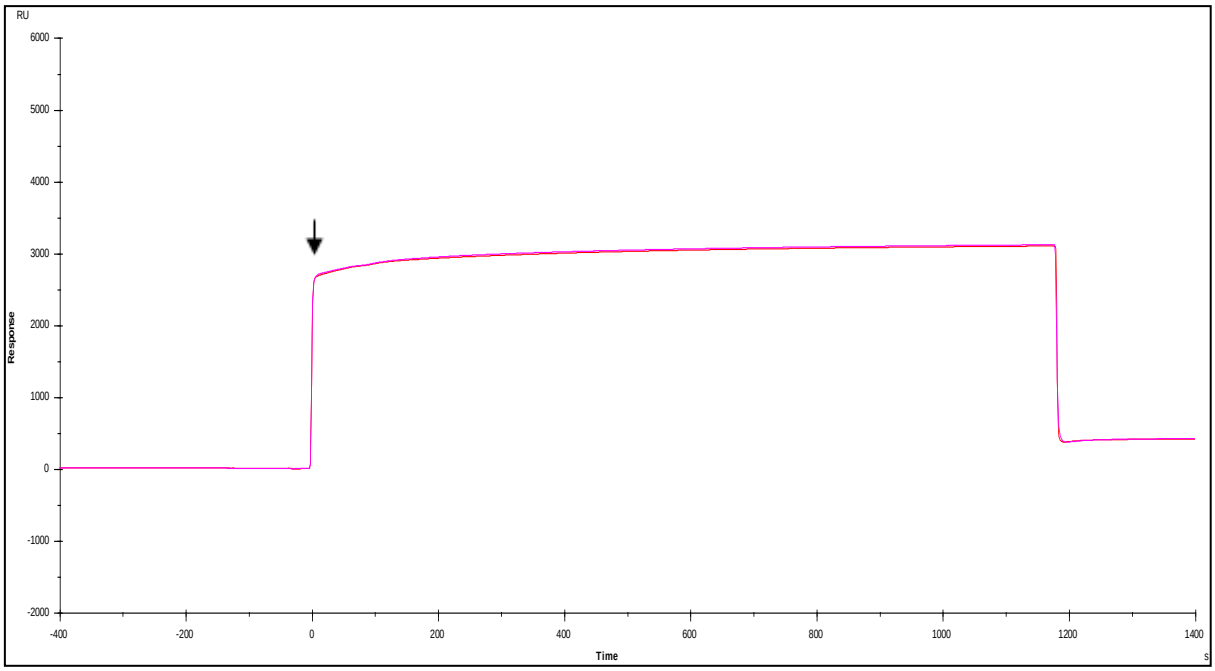

Figure 21. SPR sensogram of addition of CD82 (0.5%). DPC to NiNTA chip. Magenta, nickel coated flow cell. Red, uncoated flow cell. CD82 loading gave identical responses for both nickel coated and uncoated flow cells indicating non-specific binding. ↓, addition of CD82, 0.5% DPC.

3.4 Solid State NMR

Solid state NMR (ssNMR) is advantageous for studying membrane proteins as it does not require the protein to be soluble. Therefore, ssNMR was used to observe the interaction between CD82 and GM2. Preliminary preparations of POPC and POPC with GM2 were analysed by ssNMR to check that GM2 could be observed in the samples. The spectra for POPC and POPC with GM2 were almost identical, indicating that the peaks observed were for POPC and GM2 was not detected (fig. 22). Alternative methods of sample preparation for ssNMR analysis are being trialled.

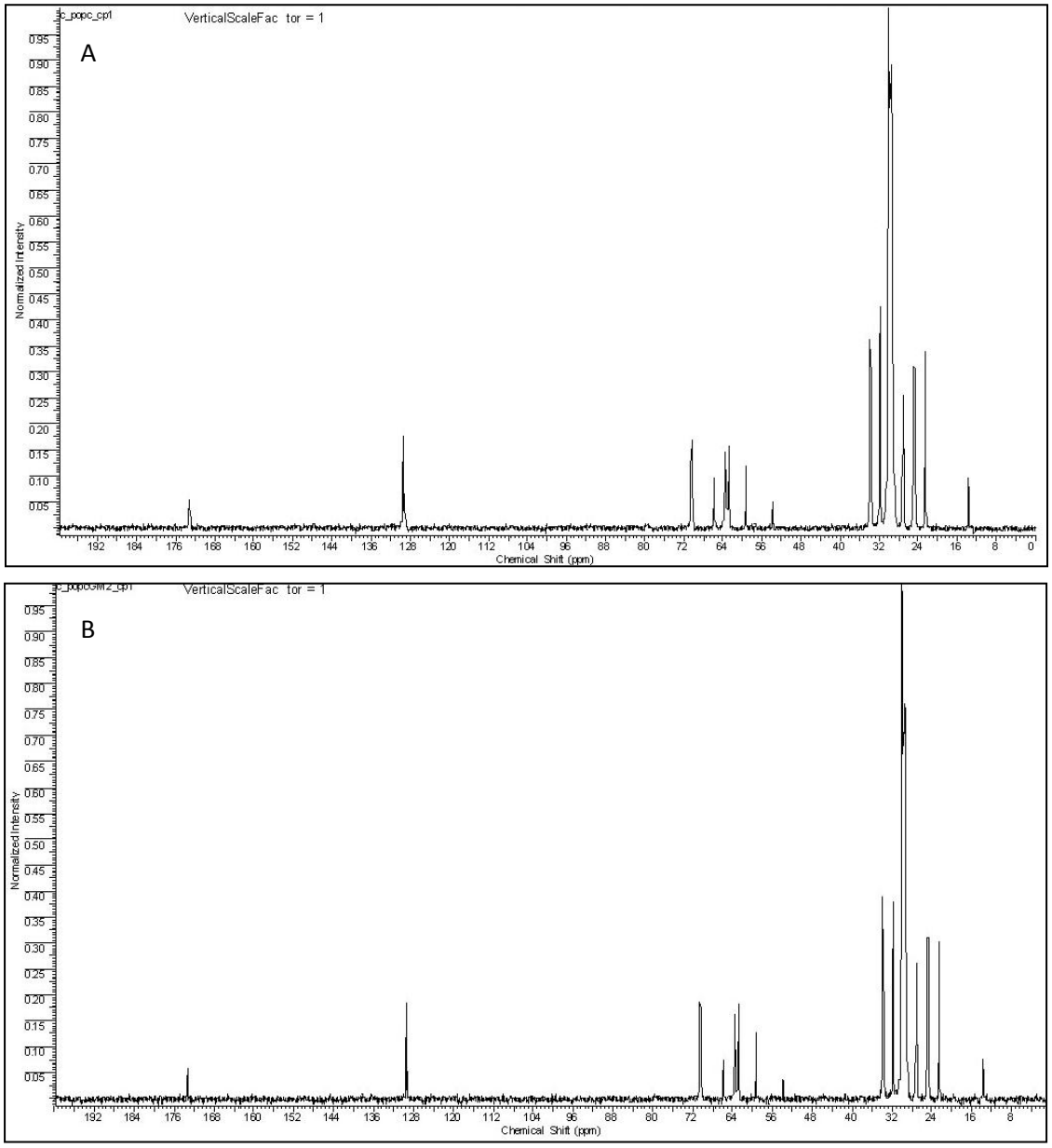

Figure 22. SsNMR spectra of POPC and POPC-GM2 micelles. A. POPC micelles. B. POPC-GM2 micelles. Spectra for both POPC and POPC-GM2 were identical, indicating that GM2 was could not be detected.

4. Discussion

Transmembrane proteins are notoriously difficult to purify using recombinant methods and protein yield is often very low. As a result, research into this field has lagged behind others. As transmembrane proteins are embedded in the lipid bilayer and are hydrophobic, they are poorly soluble in aqueous conditions and can become unstable, losing their conformation and functionality (30). Transmembrane proteins may be solubilised in detergent. However, this can affect protein function and interfere with certain biological assays and prove problematic for structure determination using x-ray crystallography or NMR. These issues were also encountered in in this study. Although β-OG and DPC were used to solubilise the CD82 by masking the hydrophobic transmembrane regions of CD82, they also bound to the hydrophobic surface of the ELISA plate. Despite this, the interaction between CD82 and GM2 was still observed by ELISA. Attempts to confirm this interaction by surface plasmon resonance were unsuccessful while efforts to observe the CD82-GM2 interaction by ssNMR are on-going. This data also suggests that full length CD82 protein expressed in yeast has similar activity to CD82 expressed in mammalian systems.

These results support research by Odintsova et al and Todeschini et al showing that CD82 binds to gangliosides, in particular GM2, and this interaction is important for CD82 function (25, 26). It is known that CD82 in the presence of GM2 inhibits phosphorylation of tyrosine residues in C-Met to inhibit cell proliferation and motility in response to HGF binding to the cMet receptor and also inhibits HGF independent cMet signalling via interaction with integrins. In cancer cells, CD82 is downregulated, so that inhibition of cMET is relieved, promoting metastasis (27). It is unclear why GM2, with its apparently anti-metastatic activity, is elevated in cancer. It has been observed that GM2 is upregulated in melanoma and increases tumour growth and metastasis (31). This contrasts with the anti-metastatic effects observed on cMet in complex with CD82. Elevation of gangliosides in tumour cells may be as a result of cells gaining a cancer phenotype or may be

part of a compensatory or negative feedback mechanism in an attempt to regain control of cell motility. This is a point for further research. It is possible that low levels of GM2 facilitate anti-metastatic activities of CD82, while upregulation of GM2 could alter TEM composition and compartmentalisation of other membrane components to modify cell behaviour to a metastatic phenotype. Alteration of TEM composition may favour different combinations of molecular interactions and result in activation of different signalling pathways. It has been demonstrated that depletion of GD1a alters CD82 distribution on the cell surface (26). It may be interesting to observe if depletion of GM2 has a similar effect on CD82 and would indicate the role of GM2 in CD82 compartmentalisation.

Ganglioside GM2 has previously been highlighted as a possible target for cancer therapeutics and there have been several attempts to use anti-GM2 antibodies to disrupt GM2 activity. A study by Hanibuchi et al 1998 using chimeric anti-GM2 antibodies showed promising results in vivo by inducing antibody dependant cytotoxicity and reducing tumour metastasis (32). However, the use of chimeric antibodies is limited due to the risk of adverse effects. More recently, Yamada et al 2011 have produced a humanised anti-GM2 antibody which reduced metastasis and triggered apoptosis in a mouse model of small cell lung carcinoma (33). Two of the monoclonal anti-GM2 antibodies used in this study, BIW 8962 and KM8927 are currently in clinical trials for the treatment of melanoma. With these promising prospects, an intriguing aspect is the role of CD82 in this situation. It is possible that the anti-metastatic effects of these antibodies may be related to the interaction of GM2 with CD82. This raises CD82 as a possible target for cancer therapeutics, and a better understanding of this interaction may lead to the production of more specific anti-GM2 antibodies inhibiting the pro-metastatic activities of GM2 while conserving the anti-metastatic interaction with CD82.

While using the full length CD82 protein posed problems with the use of aqueous solutions and maintaining protein stability, it may be possible to observe the interaction with

GM2 by using the extracellular domain of the tetraspanin. Tetraspanin extracellular domains, specifically the large extracellular loop, have been demonstrated to be functionally active by several studies including Monk et al 2002 who observed that the ECD of CD82 and several other tetraspanins could inhibit infection of macrophages by HIV (human immunodeficiency virus) (34).

An alternative approach to overcome the problems posed by detergents would be to use nanodiscs, originally developed by Sligar et al(35). Nanodiscs consist of phospholipids held together into a bilayer by membrane scaffold protein which shields the hydrophobic region of the bilayer from aqueous solution. Membrane proteins solubilised in detergent can be embedded into the nanodisc and the detergent subsequently removed. This maintains protein stability and also has the advantage of mimicking the structure of the plasma membrane allowing proteins to be studied in an environment that more closely resembles their natural state. These nanodiscs can be bound to NiNTA sensor chips and used in SPR for binding studies.

The interaction between CD82 and GM2 has important implications on our understanding of the role of CD82 in cancer metastasis. This study demonstrated the CD82-GM2 interaction by ELISA while SPR and ssNMR approaches require refinement in order to confirm this observation. The functional significance of this interaction must also be explored. In particular, the apparently contrasting role of GM2 and CD82 in metastasis needs further investigation. Although anti-GM2 antibodies are in development, a greater understanding of the role of GM2 and its interaction with CD82 could lead to the design of a more effective anti-metastatic treatment.

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