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Possible Interacting Proteins for Retinol Binding Protein and Cellular Retinol Binding Protein

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ABSTRACT

Retinol or vitamin A is crucial for many biological processes in the body such as proliferation, differentiation and reproduction. It is mainly stored in the liver and can be broken down to retinoic acid inside the cell. It travels in the circulatory system attached to Retinol Binding Protein (RBP) and its transport into the cell is mediated by the RBP receptor STRA6. Inside the cell, retinol binds to cellular Retinol Binding Protein (CRBP) and can be converted to retinoic acid, which can then act as a regulator of transcription. High levels of RBP have been implicated in type 2 diabetes. Looking for possible interacting partners for proteins such as RBP, can further elucidate the function of the protein and its possible role in type 2 diabetes. The Membrane Yeast Two Hybrid (MYTH) system allows for the study of possible interacting partners for membrane proteins. Previous studies using (MYTH) looked at possible interacting partners for RBP and CRBP. Two hits for RBP were G-protein coupled receptors (GPCRs) RAIG2 and RAIG3. One hit for CRBP was the TRPC4 ion channel. Retinoic acid inducible gene (RAIG) is a recently discovered family C GPCR and the TRPC4 channel is a membrane ion channel responsible for calcium and sodium ion influxes into the cell. An RBP-RAIG interaction could implicate the RAIG receptor in type 2 diabetes. A CRBP-TRPC4 interaction could activate the ion channel causing a calcium influx and a possible mechanism of insulin resistance in the cell, the genesis of which is still very controversial. This thesis involves performing a series of pull down assays using a novel oil-based assay to determine if the proteins in the yeast two hybrid system are indeed interacting.

List of Abbreviations

ATRA All-*trans*-Retinoic Acid

BSA Bovine Serum Albumin

BCA Bicinchoninic Acid

bp Base Pair

CAPS *N*-cyclohexyl-3-aminopropanesulfonic acid

CHAPS 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate

CMV Cytomegalovirus

CRBP Cellular Retinol-Binding Protein

DDK-tag 8 Amino Acid Affinity Tag, Sequence DYKDDDDK

DDM Dodecylmaltoside

DNA Deoxyribonucleic acid

DTT Dithiothreitol

ECL Enhanced Chemiluminescent

FCS Foetal Calf Serum

FLAG®-tag (registered trademark of Sigma Aldrich, see DDK-tag)

GPCR G Protein-Coupled Receptor

GST Gluthathion-S-transferase

HA Haemagglutinin

HEK293 Human Embryonic Kidney Cells

His-tag 6 Amino Acid Affinity Tag, Sequence HHHHHH

HRP Horseradish Peroxidase

IgG Immunoglobulin G

IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani Medium
MW	Molecular Weight
Myc-tag	10 Amino Acid Affinity Tag, Sequence EQKLISEEDL
NFDM	Non-Fat Dry Milk
Ni-NTA	Nickel-Nitrilotriacetic Acid
ORF	Open Reading Frame
O/N	Overnight
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PBS	Phosphate Buffered Saline
PBS-t	Phosphate Buffered Saline (0.05 % (v/v) Tween-20)
PVDF	Polvinylidene Fluoride
RAR	Retinoic Acid Receptor
RAIG	Retinoic Acid Inducible Gene
RBP	Retinol-Binding Protein
R/T	Room Temperature
RXR	Retinoid X Receptor
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
STRA6	Stimulated by Retinoic Acid Gene 6
TAE	Tris-Acetate-EDTA
TEMED	N,N,N',N – Tetramethylenediamine
TM	Transmembrane

TRP Transient Receptor Potential

TTR Transthyretin

Units

µg Micrograms

µl Microlitres

g Gram

kDa Kilodalton

M Molar concentration

mg Milligram

ml Millilitre

mM Millimolar concentration

ng Nanogram

nm Nano Meter

rpm Revolutions per minute

U Units

v Volts

Chapter 1

Introduction

1.1 Vitamin A

Retinol or vitamin A is important for many processes in the body, such as differentiation, proliferation, vision and reproduction (Olsen, 1996). Retinol is stored mainly in the liver as retinyl esters but transported round the body as retinol (Quadro *et al*., 1999). When it reaches its target tissue, it is usually converted into all-trans-retinoic acid (ATRA), except in certain eye cells where the aldehyde retinal is produced for the visual pigment rhodopsin (Quadro *et al*., 1999). ATRA acts as a transcription regulator controlling the transcription of hundreds of genes responsible for mammalian reproduction and development (Quadro *et al*., 1999). ATRA travels to the nucleus where it binds heterodimeric nuclear receptors such as retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Giguere *et al*., 1987; Petkovich *et al*., 1987; Chambon, 1996). These receptors are ligand dependent transcription factors and can now control the expression of over 300 genes, mainly responsible for reproduction, proliferation and differentiation (Quadro *et al*., 1999).

An important metabolite of retinol is 11-cis-retinal which is the chromophore responsible for photon absorption in GPCR rhodopsin (Palczewski, 2006). A conformational change occurs in the protein, following absorption of a photon of light by 11-cis-retinal, causing isomerisation to the all-trans form (Stryer, 1991). This conformational change initiates the visual transduction pathway (Stryer, 1991).

Vitamin A importance to the body is manifested by a series of pathological conditions that occur through deficient or excessive exposure to retinoids (Underwood, 1994; Mark *et al*., 2006). Blindness and night blindness in children is caused by Vitamin A deficiency (VAD) (Dowling, 1966), while osteoporosis has had links with high concentrations of vitamin A (Zile, 1998). Due to these diseases, it is important that the correct concentration of retinol is

delivered to target tissue in the body (Redondo *et al*., 2006). Retinol binding protein (RBP) facilitates the transport of retinol from the liver to target tissue, thus maintaining the homeostasis of retinol in the body (Redondo *et al*., 2006). RBP also prevents retinol partitioning into the membrane due to retinols hydrophobic nature (Naylor and Newcomer, 1999).

1.2 Retinol Binding Protein

Retinol Binding Protein (RBP) is a lipocalin and has a molecular weight of 21kDa (Redondo *et al*., 2006). Predominately synthesised in the liver, it has also been synthesised in the gonads, kidneys, adipocytes and retinal pigment epithelium (Redondo *et al*., 2006). RBP delivers retinol to almost all tissues (Kawaguchi *et al*., 2008) and secretion of RBP from the liver only occurs upon retinol binding (Ronne *et al*., 1983). Holo-RBP binds to the tetrameric thyroxine carrier protein, transthyretin (TTR), forming a macromolecular complex (Navab *et al*., 1977) which is then secreted from the liver (Melhus *et al.,* 1991; Episkopou *et al*., 1993). There is one molecule of retinol bound to the RBP protein in the TTR tetramer complex secreted from the liver (Gottesman *et al*., 2001). Holo-RBP circulates in the bloodstream as a monomer mostly bound to the tetramer of TTR, the combined complex having a molecular weight of 77kDa (Redondo *et al*., 2008). The Holo-RBP-TTR complex is important as it prevents RBP-retinol being eliminated by glomerular filtration in the kidneys, thereby allowing RBP to deliver retinol to the target cells (Goodman, 1984; Monaco, 2000). It also encapsulates retinol in the complex preventing the hydrophobic molecule being partitioned in the membrane (Redondo *et al*., 2008). TTR is not known to effect RBP delivering retinol to the cell, but does prevent renal filtration of RBP in the kidneys (Gottesman *et al*., 2001). After retinol is delivered to the target cells, the apo-RBP-TTR complex is unstable and RBP is excreted by the kidneys (Redondo *et al*., 2006).

1.3 STRA6

Kawaguchi *et al*., 2007, identified STRA6 as a widely expressed high-affinity, RBP specific receptor. The receptor has a molecular weight of 74kDa and is the retinoic acid-stimulated gene (Kawaguchi *et al*., 2008), previously found in P19 carcinoma cell line (Bouillet *et al*., 1997). The receptor is a multi-transmembrane, hydrophobic protein, which binds to retinol-RBP (holo-RBP) with high affinity, resulting in the transport of retinol into the cell (Sivaprasadarao and Findlay, 1988, Kawaguchi *et al*, 2007). STRA6 binds to the monomer RBP, encapsulating the retinol, meaning holo-RBP dissociates from the TTR complex before binding to STRA6 (Sivaprasadarao and Findlay, 1988). Due to the importance of retinoids in the developmental process, STRA6 is highly expressed during embryonic development but expression is also high in adult organ systems particularly cells comprising blood-organ barriers (MacDonald *et al*., 1990; Smeland *et al*., 1995). The membrane topology of STRA6 contains nine putative transmembrane domains interrupted by a large intracellular loop, a larger intracellular loop, an extracellular N-terminus and an intracellular C-terminal tail (Redondo *et al*., 2008). Due to the large size of the intracellular tail it seems reasonable to suggest that this region interacts with possible intracellular binding partners (Redondo *et al*., 2008). How retinol gets into the cell is unknown after it is taken up from RBP (Redondo *et al*., 2008). When RBP binds to STRA6, it is likely that STRA6 binds to cellular retinol binding protein (CRBP), which is the only known molecule that binds retinol in the cell (Redondo *et al*., 2008).

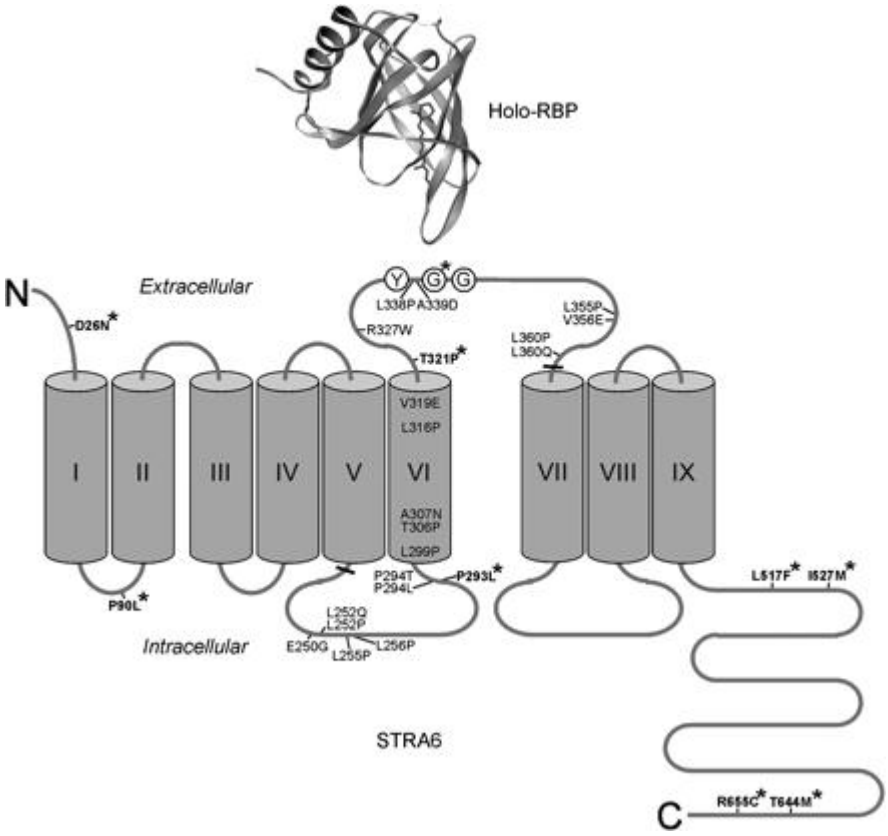


Figure 1.1: The Membrane Topology of STRA6.

Protein contains 9 transmembrane regions, with an extracellular N-terminus and a large intracellular C-terminal tail. The protein contains a large extracellular loop between helix 6 and 7 and also 2 intracellular loops which could be involved with binding to RBP and CRBP respectively. The diagram also shows the structure of holo-RBP with the one molecule of retinol attached. (Kawaguchi *et al*., 2008).

1.4 CRBP

The hydrophobicity of retinol means that it is bound to one of several cellular retinol binding proteins (CRBP) in the cell (Piantedosi *et al*., 2005). CRBP protects retinol from becoming partitioned into the membrane and disrupting normal cellular activity (Piantedosi *et al*., 2005). The main role of cRBP in retinol uptake from STRA6 is to facilitate the transport of retinol to specific enzymes which convert it to the stored esterified form (LRAT) or oxidise it to retinoic acid (Piantedosi *et al*., 2005). An enzyme called retinol dehydrogenase converts retinol attached to CRBP into retinal (Gottesman *et al*., 2001). The retinal can then get broken down by retinal dehydrogenase into retinoic acid, which acts as a regulator of transcription factors in the nucleus (Gottesman *et al*., 2001). Some doubt has been cast on this process because newborn mice lacking CRBP-I still show expression of retinoic acid responsive gene RARβ2 in the liver (Gottesman *et al*., 2001). There are three known CRBP molecules, I, II and III (Piantedosi *et al*., 2005). CRBP-I is highly expressed in the liver where it is needed for the storage of retinoid as retinyl esters (Piantedosi *et al*., 2005). Mice lacking CRBP-I are unable to store retinoids in the liver and develop symptoms of retinoid deficiency (Piantedosi *et al*., 2005). CRBP-II is expressed solely in the intestines but at a high level and is mainly involved with uptake and storage of retinol as retinyl esters, mice lacking CRBP-II showing a decrease in retinoid absorption (Piantedosi *et al*., 2005). CRBP-III facilitates retinyl esters storage in milk, with retinyl esters being reduced in milk in mice lacking CRBP-III (Piantedosi *et al*., 2005). Lecithin: retinol acyltransferase (LRAT) and acyl CoA: retinol acyltranferase (ARAT) are the two enzymes that catalyse retinyl ester synthesis (Gottesman *et al*., 2001). These findings suggest that CRBP is critically important for storing retinol as retinyl esters but may not be involved with the production of retinoic acid and it's translocation into the nucleus (Gottesman *et al*., 2001).

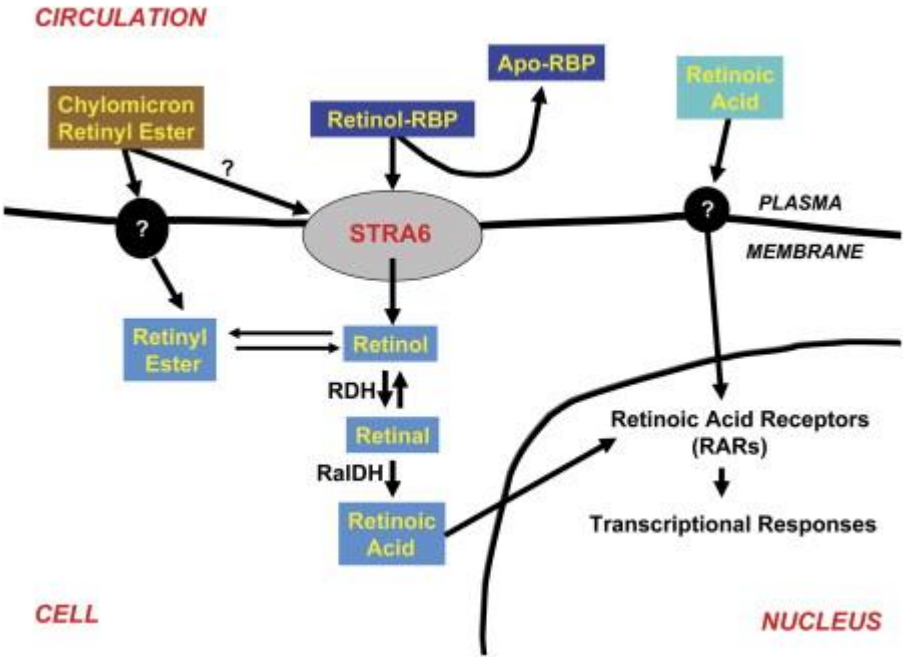


Figure 1.2: Retinoid Metabolism

This diagram shows the progress of holo-RBP after it is excreted from the liver bound to TTR. Holo-RBP binds STRA6 in the plasma membrane. STRA6 facilitates the transport of retinol into the cell. The retinol bound to CRBP inside the cell is either broken down to retinoic acid or stored in the cell as retinyl esters. Retinol can get broken down to retinal by retinol dehydrogenases (RDH). The retinal can get broken down to retinoic acid by retinal dehydrogenases (RalDH). Retinoic acid travels into the nucleus and binds to Retinoic Acid Receptors (RARs). These receptors are ligand dependent transcription factors and can now control the transcription of many genes (Blaner 2007).

1.5 Type 2 Diabetes

Type 2 diabetes is a debilitating, chronic condition caused by multiple tissues becoming resistant to insulin, which results in elevated glucose levels in the blood plasma called

hyperglycaemia (Zimmet *et al*., 2001). Insulin resistance is a strong and early indicator of the subsequent development of type 2 diabetes (Graham *et al*., 2006). A major cause of type 2 diabetes is impaired insulin action in adipose tissue, skeletal muscle and liver (Yang *et al*., 2005). Cells become resistant to the action of insulin and the pancreatic beta cells cannot compensate for this resistance by producing enough insulin (Graham *et al*., 2006). In time, the pancreatic beta-cells become exhausted and the supply of insulin drops, producing type 2 diabetes (Graham *et al*., 2006).

The RBP-STRA6 interaction has recently been implicated in insulin resistance (Yang *et al*., 2005), with serum RBP4 levels elevated in type 2 diabetes patients (Klöting *et al*., 2007). A landmark paper by Yang et al., 2005 chronicled the first evidence for a direct role for RBP in obesity, insulin resistance and type 2 diabetes as follows:

- RBP is elevated in insulin resistance mice and in humans with obesity and type 2 diabetes.
- Fenretinide (an anti-cancer synthetic retinoid), which accelerates RBP urinary excretion (and hence reduces serum levels), reduced insulin resistance.
- Rosiglitazone (antidiabetic drug) reduced serum RBP levels and increased insulin sensetivity in obese mice.
- Insulin sensitivity was enhanced in RP knock-out mice.
- RBP over-expression in transgenics or RBP injection in normal mice caused insulin resistance.
- There were no differences in glucose, free fatty acids, leptin, adiponectin or resistin levels in RBP over-expressing mice, indicating that RBP was not acting through any of these factors.
- In RBP over-expressing-mice, skeletal muscle insulin-induced PI(3)Kinase was reduced.

- RBP injection into normal mice reduced both insulin-stimulated PI(3)Kinase (PI3K) activity and the phosphorylation of insulin receptor substrate (IRS1) at the tyrosine (612) associated with the docking of the p85 subunit of PI3K.
- Fenretinide restored IRS1 phosphorylation levels in skeletal muscle cells.
- The source of additional RBP, which otherwise is under tight control via the liver, is thought to be adipose tissue meaning obesity is presumed to result in elevated levels of RBP through secretion from adipose tissue.
- This elevated RBP in turn acts on peripheral tissues such as skeletal muscle to attenuate insulin sensitivity as indicated by reduced IRS1 phosphorylation and PI3K activity.

Since TTR prevents the renal clearance of RBP, it was no surprise to see TTR levels also elevated in Type 2 diabetes patients (Klöting *et al*., 2007).

An interesting recent paper by Berry and Noy (2011), claims that holo-RBP and STRA6 could have a role in the STAT-JAK signaling pathway. The paper showed:

- Holo-RBP binding to STRA6 induces the phosphorylation of a tyrosine residue in the receptors C-terminus, thereby activating a JAK/STAT signaling cascade.
- In STRA6 expressing cells such as adipocytes, holo-RBP induced the expression of STAT target genes, such as SOCS3 and PPAR*y*.
- SOCS3 is known to supress insulin signalling and PPAR*y* enhances adipose lipid storage
- Holo-RBP binding to STRA6 regulates gene expression to inhibit insulin signalling and enhance lipid accumulation.

Glucose-transport 4 (GLUT4) is the main insulin stimulated glucose transporter (Graham *et al*., 2006). GLUT4 removes glucose from the circulatory system and is a key regulator in

whole body glucose homeostasis (Huang and Czeach., 2007). GLUT4 is predominantly intracellular in the unstimulated state, with translocation to the plasma membrane induced by certain stimuli such as insulin (Huang and Czeach., 2007). The expression of GLUT4 in adipose tissue is reduced in the insulin resistance state and it is now believed that the reduction in expression and translocation of GLUT4 is caused by RBP (Yang *et al*., 2005). Elevated levels of RBP are found in adipocyte-specific GLUT4 knockout mice (Yang *et al*., 2005). This has led to the claim that elevated levels of serum RBP could be responsible for insulin resistance (Yang *et al*., 2005). It is also considered that there is a direct link between elevated levels of RBP and downregulation of the GLUT4 transporter in adipocytes (Yang *et al*., 2005).

1.6 RAIG (Retinoic acid-inducible gene)

Retinoic acid-inducible gene 1 (RAIG1) was first identified by Cheng and Lotan (1998) using differential display, with RNA isolates from untreated and all-trans-retinoic acid (ATRA) treated human oral squamous carcinoma cell lines. The receptor's name came from the fact that these receptors were upregulated *in vitro* by retinoic acid (Cheng and Lotan, 1998).

Subsequently Osborne and Larsen (2000) revealed sequence and expression information on GPRC5B (RAIG2) by homology searching of additional subtypes to family C G-protein coupled receptors (GPCRs) such as the human metabotropic glutamate receptor subtype 2. RAIG2 mRNA expression levels were high in kidney, pancreas and testis (Osborne and Larsen, 2000) while RAIG1 mRNA expression levels were high in the lung (Cheng and Lotan, 1998). Both transcripts show high sequence similarity but are expressed in different levels in different cell lines (Osborne and Larsen, 2000). High levels of RAIG2 mRNA were discovered in rat brain and spinal column which is in general agreement with human

distribution (Robbins et al., 2002). Western blot analysis of RAIG2 showed a band at 68kDa suggesting post translational modification as RAIG2 has a predicted protein molecular mass of 50kDa (Robbins et al., 2002).

Robbins *et al*., 2000, discovered GPRC5C (RAIG3), using homology searching against the Genbank sequence database using the *caenorhabditis elegans* metabrotropic receptor as the search sequence. The sequence for RAIG1, RAIG2 and RAIG3 showed high sequence similarity to other family C GPCRs, especially in their transmembrane regions (Robbins et al., 2000). Family C GPCRs are a sub-family of the GPCRs, defined by transmembrane sequence similarity, which largely contains receptors for neurotransmitters, glutamate and c-aminobutyric acid (GABA), receptors for calcium, some taste and pheromone molecules, as well as some orphan receptors (RAIGs) (Pin *et al*., 2004). The N-terminus is usually very large (over 600 amino acids) and is the part of the protein mostly responsible for ligand binding (Pin *et al*., 2004). The N-terminal domains of the RAIGs differ to the other family C GPCRs as they are significantly smaller (Robbins et al., 2000). This may mean a possible ligand binding region for the receptors may lie in the transmembrane region as opposed to the N-terminal region (Robbins et al., 2000). The RAIGs also lack certain residues that are conserved among all family C GPCRs (Pin *et al*., 2003). These residues include 2 cysteines that link the top of TM3 and the second extracellular loop, the highly conserved Trp in TM6, and the conserved FNEAK motif at the bottom of TM6 (Pin *et al*., 2003). Similar to RAIG1 and RAIG2, expression of RAIG3 mRNA increased in cells treated with ATRA suggesting an ability of retinoic acid to regulate GPCR signalling (Robbins et al., 2000). RAIG3 mRNA expression levels were high in selective brain areas such as the cerebellum and also both mesoderm and epithelial derived tissues (Robbins et al., 2000). The discovery of an agonist would give great insight into the function of these novel receptors, but the difference in

structure and a possible binding site (small N-terminal), to other family C GPCRs complicates the picture (Osborne and Larsen, 2000).

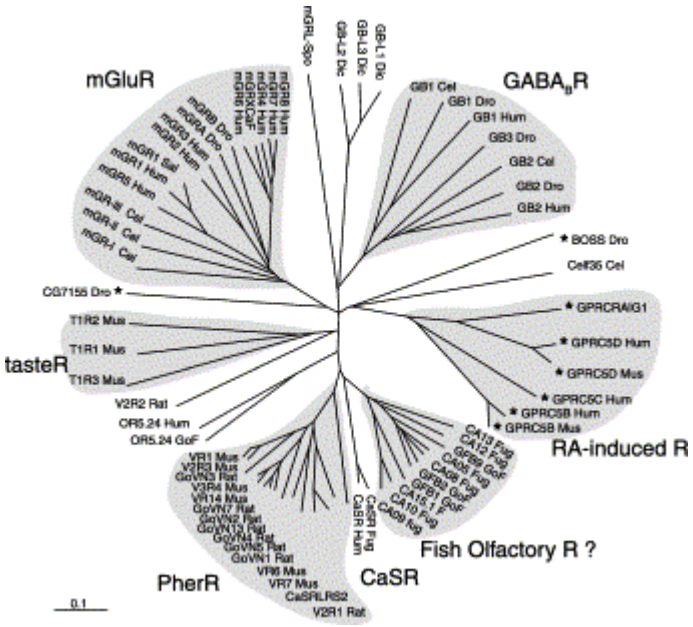


Figure 1.3: Phylogenetic Tree of Family C GPCRs.

The tree shows the recently identified RA (Retinoic acid) induced receptors which include the RAIGs. Other groups include the metabotropic glutamate receptors, putative pheromone receptors, sweet and amino acid taste receptors, GABA receptors and the Fish olfactory receptors. (Pin *et al*., 2003)

1.7 TRP (Transient Receptor Potential) Ion Channels

The TRP ("transient receptor potential") ion channel consists of a huge superfamily that comprises more than 30 channels (Pedersen *et al*., 2005). There are 7 main subfamilies: the TRPC ('Canonical') family, the TRPV ('Vanilloid') family, the TRPM ('Melastatin') family, the TRPP ('Polycystin') family, the TRPML ('Mucolipin') family, the TRPA ('Ankyrin')

family, and the TRPN ('NOMPC') family (Pedersen *et al*., 2005). All TRP channels are cation channels and contain 6 transmembrane domains, with their N and C-terminus on the intracellular side (Pedersen *et al*., 2005). Its pore which is highly selective for sodium and/or calcium ions is located between transmembrane 5 and 6 (Clapham *et al*., 2003). They are thought to possess a similar architecture to voltage –gated K channels (Clapham *et al*., 2003). This characteristic 6 transmembrane architecture is the only common/homologous feature contained in all the TRP subfamilies (Clapham *et al*., 2003). TRPC channels contain a 25 amino acid motif called the TRP box on the C-terminus, which is not a feature of any other TRP subfamily (Clapham *et al*., 2003). TRPC and TRPV channels contain ankyrin repeats on their N-terminal domain, while proline rich regions are observed on the C-terminal region of TRPC and TRPM channels (Clapham *et al*., 2003). The C-terminus of TRPC4 and TRPC5 contain a PDZ binding motif which is not seen in any other TRP channel (Clapham *et al*., 20023). The PDZ region of TRPC4 and TRPC5 can interact with PDZ domain containing proteins such as the sodium-hydrogen exchanger regulatory factor as well as phospholipase Cβ (Pedersen *et al*., 2005).

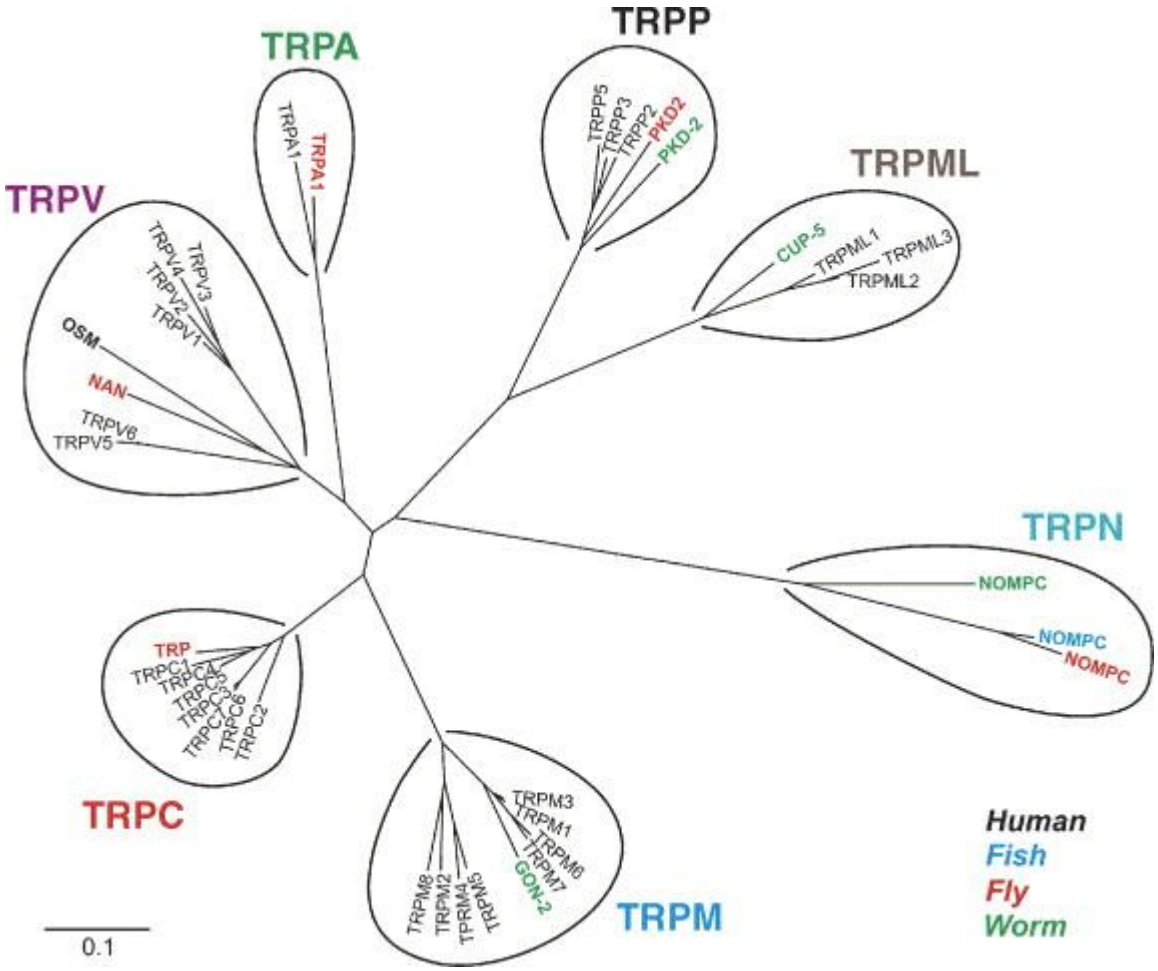


Figure 1.4: The Phylogenetic Tree of the TRP Superfamily.

The diagram shows how big the TRP family is and also its expression in many different animal types (Pederson et al., 2005).

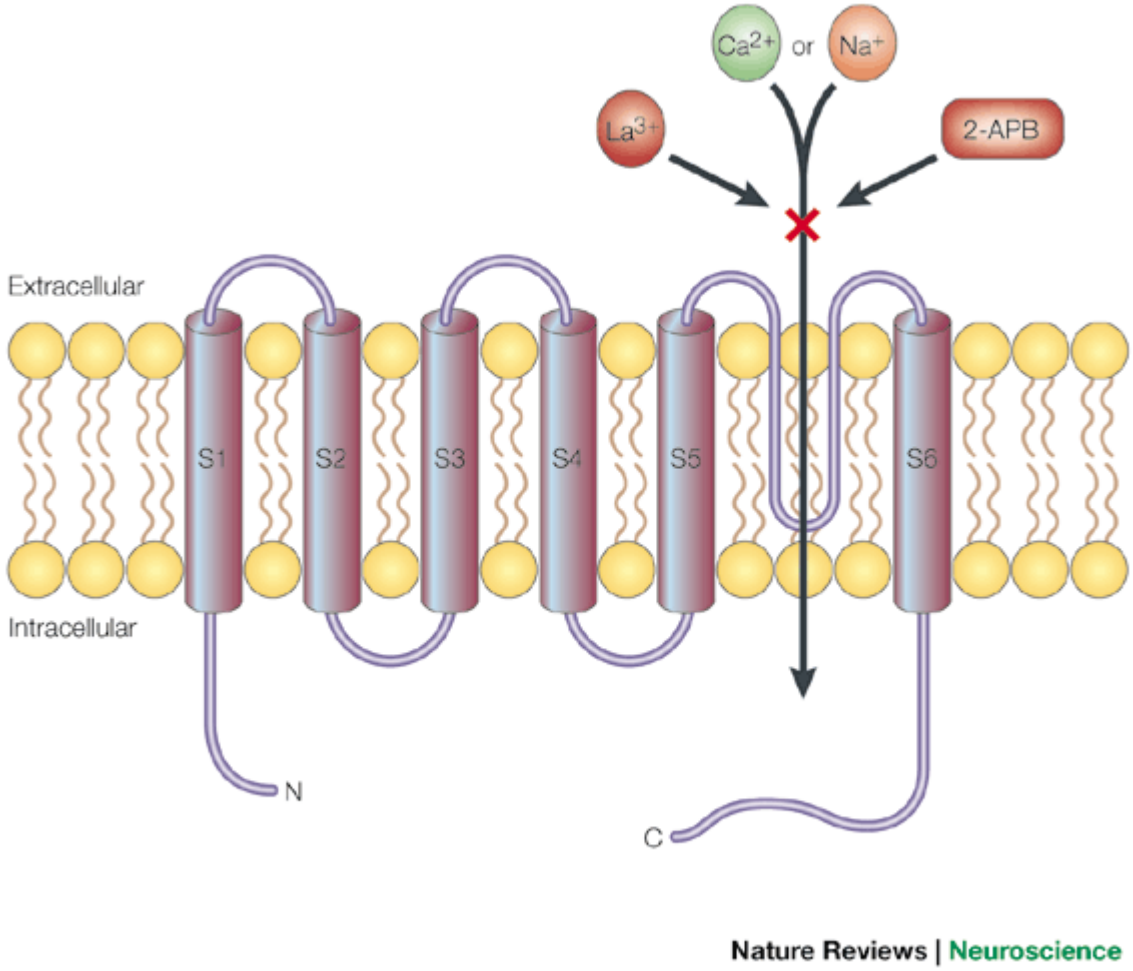


Figure 1.5: Membrane Topology of TRP channels.

TRP channels comprise a 6 transmembrane region with the pore contained between helix 5 and 6 (S5 and S6). The diagram shows that both lanthanum (La^{3+}) and 2-aminodiethyldiphenyl (2-APB) can block these channels. The channel pore is selective for calcium (Ca^{2+}) and sodium (Na^{+}) ions. The N and C-terminus are both intracellular and both interact with many different intracellular proteins (Clapham *et al*., 2001).

Activation mechanisms for TRP channels are numerous, ranging from intra- and extracellular messengers, osmotic stress and intracellular calcium stores (Pedersen *et al*., 2005). The TRPC subfamily has been shown to interact with cadmodulin, the IP_3 receptor and the metabrotropic glutamate receptor in neurons (Pedersen *et al*., 2005). TRP channels contain many possible regions for protein-protein interactions and among the many subfamilies, their binding partners and binding regions vary greatly (Pedersen *et al*., 2005). The interaction sites in TRPC channels for protein-protein interactions are contained mostly in their intracellular N and C-terminal regions (Pedersen *et al*., 2005). TRPC channels can be activated by a decrease in intracellular calcium store concentration (Yao *et al*., 2005) and have been linked to the influx of calcium ions into the cell to replenish intracellular calcium stores depleted of calcium (Clapham *et al*., 2002) by some stimulus. The release of calcium from intracellular calcium stores is modulated by G-protein coupled receptors through the activation of phospholipase C (PLC) (Clapham *et al*., 2002). PLC is known to generate inositol 1,4,5-trisphosphate (IP_3) which can activate IP3 receptor-mediated calcium release from intracellular stores (Yao *et al*., 2005). PLCβ is known to bind TRPC4 and TRPC5 and is also one of the products resulting from the enzymatic actions of PLC (Clapham *et al*., 2002) This could link TRPC channels with calcium efflux from intracellular stores and possible replenishment of these stores through TRPC channel activation (Clapham *et al*., 2002). TRPC channel's ability to interact with the IP_3 receptor further backs up this theory (Pedersen *et al*., 2005). PLC can also activate diacylglycerol DAG, which is known to bind and activate either TRPC3, 6 or 7, which is independent of calcium store depletion (Yao *et al*., 2005). Calmodulin has been shown to bind to the C-terminus of TRPV6 in a calcium-dependent mechanism (den Dekker *et al*., 2003). TRPC channels are highly expressed in the brain, endothelium, smooth and cardiac muscle cells and the lung which is in agreement with the

other TRP subfamilies as they are widely expressed in many different cell lines (Pedersen *et al*., 2005).

An example of TRP channel regulation is its role in calcium reabsorption in epithelial cells surrounding kidney or the duodenum (Nijenhuis *et al*., 2005). TRPV5 and TRPV6 seem to be able to induce an influx of calcium ions (calcium reabsorption) into epithelial cells, surrounding the kidney and duodenum, which can regulate extracellular calcium concentration (Nijenhuis *et al*., 2005). Activated TRPV channels in the epithelial cells open and allow calcium into the cell, where it binds to calcium binding proteins (calbindins) (Nijenhuis *et al*., 2005). The calbindins can channel the calcium to either calcium ATPases or the Na^{+}/Ca^{2+} exchanger (NCX1) which aid in the extrusion of calcium across the basolateral membrane (Nijenhuis *et al*., 2005). The reabsorbed calcium in the epithelial cell does not seem to effect intracellular signalling which allows for transcellular calcium fluxes (Nijenhuis *et al*., 2005). This evidence seems to show that TRPV channels have a role in the regulation of extracellular calcium concentration (Nijenhuis *et al*., 2005). Interestingly, from the viewpoint of the work described here, regulation of calcium concentration is linked to exocytosis which in turn is associated with the incorporation of proteins into the plasma membrane.

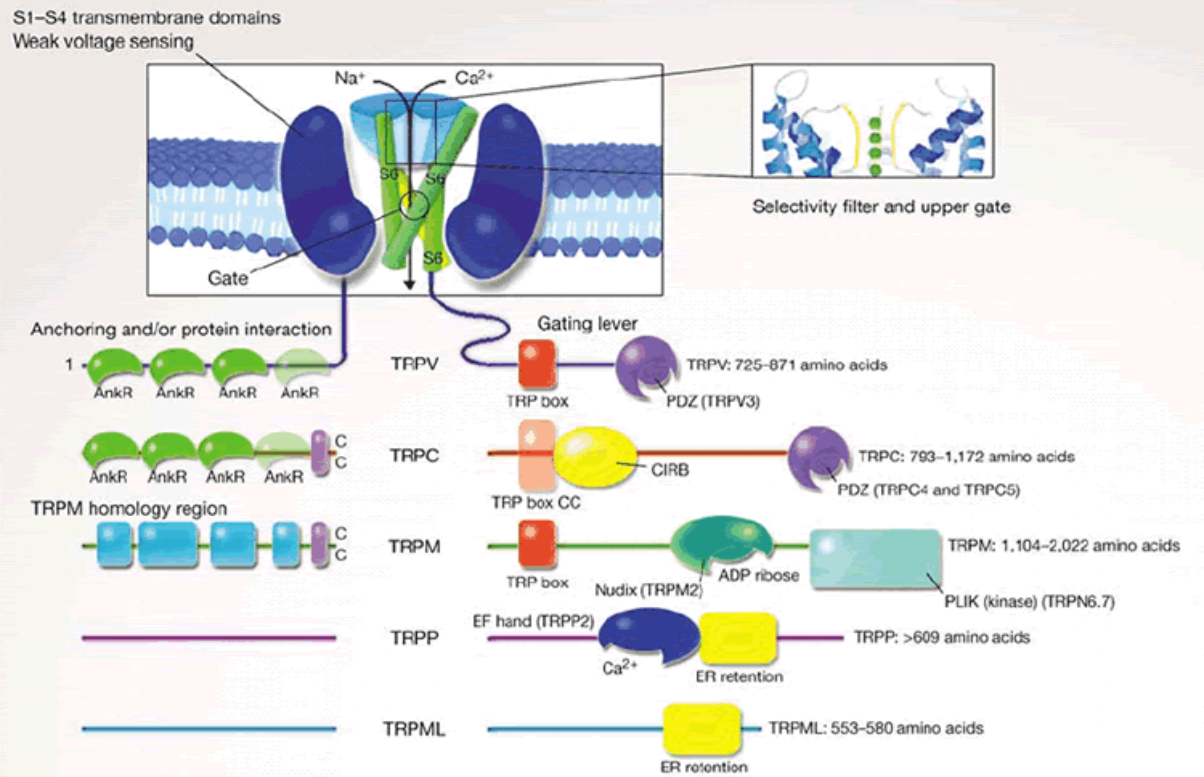


Figure 1.6: Transmembrane Domains and Protein Interaction Sites of TRP Channels

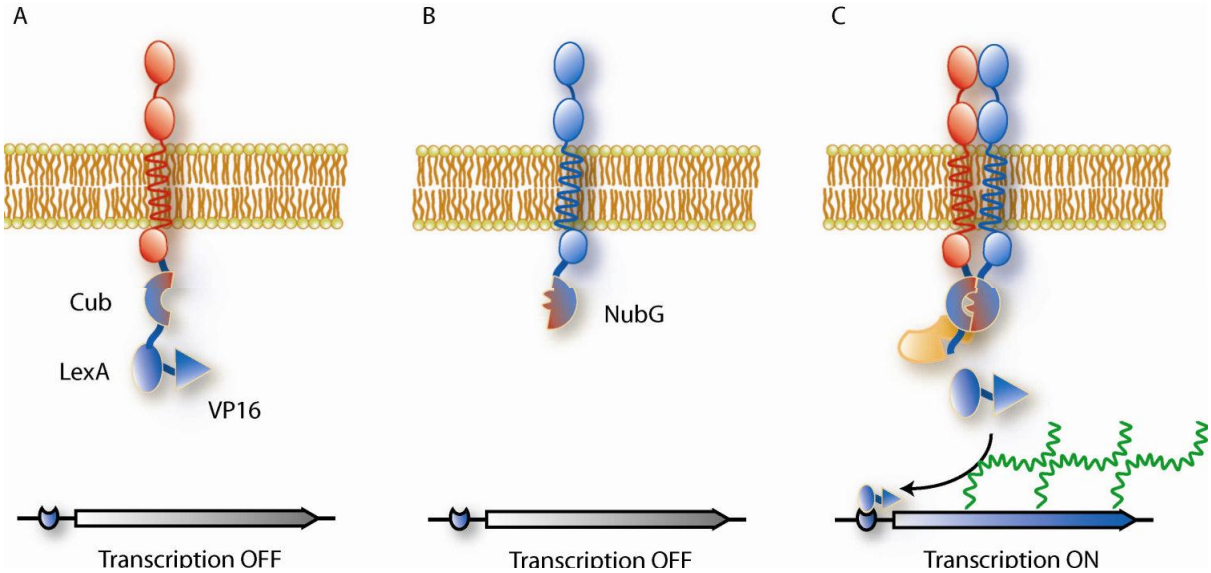
This diagram shows the pore region of the TRP channel and also the different protein binding sites contained on their C and N terminus. The pore region is formed by the fifth and sixth transmembrane regions (S5 and S6). A kink in S6 forms the gate with its cytoplasmic ends. The selectivity filter is formed by extracellular facing loops of the S5 and S6 transmembrane regions. Binding of a cation to the loops could cause a kink in S6 forcing the channel to open and the influx of cations. All TRP channel are selective for either calcium or sodium ions, except TRPV5 and TRPV6 which are Calcium selective. The intracellular regions are very diverse among the different subfamilies. Ankyrin repeats are very common on the N-terminus of TRPV, TRPC and TRPM. These 3 subfamilies also contain a TRP box sequence on their C-terminus. The C-terminus contains many binding regions for calmodulin, IP3 receptors, PDZ domain containing proteins, PLC interacting kinases and calcium binding domains (Clapham, 2003).

1.8 Membrane Yeast Two Hybrid System

The biological functions of many proteins may be determined by the identification of their interacting partners (Snider et al., 2010). Determining possible interactors for membrane proteins has proved difficult because of their hydrophobicity and location (Snider et al., 2010). The membrane yeast two hybrid system provides an effective method for determining interacting components for membrane proteins, using *Saccharomyces cerevisiae* as a host (Snider et al., 2010). The system allows full length membrane proteins to be studied in the context of the membrane and not just their soluble domains (Snider *et al*., 2010). This is unlike other yeast two hybrid systems which require the interaction to occur in the nucleus between two soluble proteins (Snider et al., 2010).

The bait and prey proteins are tagged with ubiquitin domains at their C or N terminus, which can reassociate in the cell to form pseudoubiquitin if brought into close proximity (Snider et al., 2010). The membrane protein's (bait) N or C terminus contains the C-terminal fragment of ubiquitin (C_{ub}) fused to a transcription factor containing a LexA Escherichia coli DNA-binding domain LexA and the herpes simplex virus VP16 transcriptional activation domain (Snider et al., 2010). The possible interacting proteins (prey) N or C terminal contains an N-terminal fragment of ubiquitin (N_{ub}), which has an isoleucine13 to glycine mutation, which prevents spontaneous association of N_{ub} and C_{ub} (Snider et al., 2010). If there is an interaction between the bait and prey proteins, Nub and Cub come together and form a fully folded ubiquitin (Snider et al., 2010). This fully folded pseudoubiquitin is recognised and cleaved by cytosolic deubiquitinating enzymes (DUBs), which cleave the pseudoubiquitin freeing the transcription factor (Snider et al., 2010). The transcripition factor travels to the nucleus and activates expression of a reporter gene under the control of promoters containing LexA binding sites (Snider et al., 2010). The bait and prey proteins are co-expressed in

Saccharomyces cerevisiae and expression of the reporter gene allows for their growth on selective media (Snider et al., 2010). This makes the selection of prey-bait interactions highly specific and convenient (Snider et al., 2010). Retinol binding protein (RBP) and cellular retinol binding protein (CRBP) were used as the baits in a screen of cDNA libraries (preys) from brain and muscle. In this screen, RAIG2, RAIG3 and TRPC4 Zeta were revealed as hits for possible binding partners (for RBP (RAIGs) and CRBP respectively.



(Diagram taken from the Dualsystem kit 3 user manual).

Figure 1.7: Membrane Yeast Two Hybrid System.

Diagram explaining the membrane yeast two hybrid system. (A) The integral membrane bait protein fused to Cub ubiquitin which is fused to a transcription factor containing a LexA

DNA binding domain. Transcription of the reporter gene in the nucleus is turned off. (B) The integral membrane prey protein fused to the mutated NubG ubiquitin. Transcription of the reporter gene in the nucleus is turned off. (C) An interaction between the two proteins brings NubG and Cub together forming a fully folded ubiquitin, which is cleaved by dubiquitinating enzymes (DUBS), releasing the transcription factor. The transcription factor travels into the nucleus and uses its LexA binding domain to bind to promoters containing the LexA binding domain. The VP16 transcriptional activation domain induces expression of reporter genes, which will allow for growth of the yeast on selective media and also tell you that there is an interaction between the bait and prey protein (Snider *et al*., 2010)

1.9 MembraneMax™ Cell –Free Expression

The MembraneMax^{TM} Protein Expression Kit is designed for in vitro expression of soluble membrane proteins from template DNA in a single scalable reaction. The system contains all the components for the production of recombinant membrane protein. The system only requires a construct with a T7 RNA polymerase promoter, the prokaryotic Shine-Dalgarno ribosome binding site (RBS), the ATG initiation codon, the stop codon, and the T7 terminator. The *E. coli* based MembraneMax™ expression system is designed to produce high yields of membrane proteins embedded in a planar phospholipid bilayer, surrounded by a scaffold protein (based on Apo AI) of 10 nm in diameter and referred to as nanolipoprotein particles or NLPs, (Figure 1.9). The system has many advantages over other expression systems:

- Production of recombinant membrane protein of interest from an expression construct in less than 4 hours
- Production of soluble and monodispersed membrane protein population
- Microgram to milligram quantities of membrane protein
- Easy, scalable membrane protein synthesis reactions that are amendable to high-throughput for a wide range of expression needs
- Many options for optimizing the reaction if problems arise

Taken from the **MembraneMax™ Protein Expression Manual (Invitrogen).**

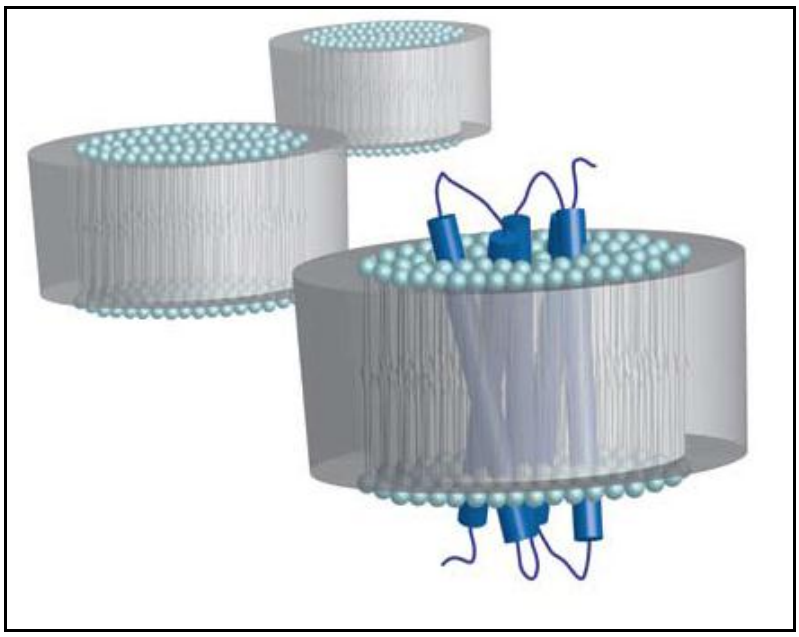


Figure 1.8: MembraneMax™ Nanolipoprotein Particles

Membrane proteins expressed in MembraneMax™ are embedded in a planar phospholipid bilayer, surrounded by a scaffold protein (based on Apo AI) of 10 nm in diameter and referred to as nanolipoprotein particles (NLPs). Shown are NLPs both with and without a membrane protein embedded. Once inserted into the lipid bilayer, the membrane protein of interest is accessible from both sides. Taken from the **MembraneMax™ Protein Expression Manual (Invitrogen).**

1.10 Aims and Objectives

The aim of this project was to follow up on these screens to determine if there were protein interactions between (i) RBP and RAIG2 (ii) RBP and RAIG3 and (iii) CRBP and TRPC4 Zeta ion channel. The project involved:

(i) Preparing molecular biology construction of these proteins which contained "tags" for identification and isolation,
(ii) Obtaining the expression of these three proteins in suitable amounts and cell systems which allowed immunopurification. And
(iii) Performing a series of pull down assays using a novel oil-based assay to determine if the proteins were indeed interacting

The possible binding of RAIG2 and RAIG3 to RBP could have serious implications in type 2 diabetes. High levels of RBP have been shown to effect insulin sensitivity (Klöting *et al*., 2007). The only receptor identified for RBP is STRA6 which is why this receptor (without proof) is implicated in the disease. If the oil pull-downs revealed an interaction with either RAIG2 or RAIG3 with RBP, this could implicate one or both of these GPCRs in the disease. It would also identify a ligand for one or both RAIG proteins thereby de-orphanise them and open up a whole to line of investigation into the role and mechanism of action of RBP.

If CRBP does bind to TRPC4, this could have important implications in the cell as TRPC4 channels have a big impact on calcium levels. The CRBP-TRPC4 interaction could activate the TRPC4 channel causing an influx of calcium into the cell. Calcium has been shown to cause GLUT4 translocation to the plasma membrane (Youn *et al*., 1991). One of the causes of type 2 diabetes is the lack of GLUT4 translocation to the plasma membrane. This possible interaction could provide a possible mechanism of insulin resistance in the cell, the genesis of which is still very controversial. It would also reveal a completely new mechanism of action of CRBP.

Chapter 2
Materials and Methods

2.1 Chemicals and Reagents

All chemicals, reagents, and proteins were purchased from Sigma unless otherwise stated. Enzymes and buffers were purchased from New England Biolabs, Promega, and Stratagene. All oligonucleotides used were purchased from Sigma Genosys.

Size markers for DNA gels were purchased from both Promega and Bioline. Plasmid DNA isolation and purification kits were purchased from Qiagen.

For protein expression; MembraneMax™ was bought from Invitrogen and *E. coli* strains from Stratagene, unless otherwise stated. Cell culture media and associated products were purchased from Gibco (Invitrogen) with the exception of FuGENE® 6 transfection reagent, obtained from Roche.

Protein purification was performed using Glutathione Sepharose™ 4 Fast Flow from GE Healthcare and/or Ni-NTA agarose, purchased from Qiagen. In the detection of proteins; ECL Western blotting substrate and 20 X BupH tank buffer were all purchased from Pierce. Molecular weight markers for protein gels were purchased from Bio-Rad. Blotting paper (3MM Chr.) was supplied by Whatman, purchased from GE Healthcare.

Antibodies were purchased from Sigma (*α*-myc-HRP and α-FLAG®), GE Healthcare (*α*-GST-HRP and *α*-rabbit-HRP), Roche (*α*-His-HRP), Dako (*α* -RBP) and Santa Cruz Biotechnology Inc. (*α* –CRBP)

2.2 Vectors, Host Strains and Cell Lines

The coding sequence for human RAIG3 was obtained in the pCMV6-Entry vector (OriGene), incorporating a C-terminal Myc and DDK-tag (shown in Figure 2.3). This vector was used in attempts to express the full length receptor in HEK293 cells and cell-free systems as

described in Chapter 3 and 4. The coding sequence of RAIG2 was subcloned into Pet-30a (+) (Novagen) (shown in Figure 2.2) for expression as a His-fusion protein for cell free expression. The coding sequence of RAIG2 was also subcloned into the pCMV6-Entry vector to express the full length receptor in HEK cells. The coding sequence for human TRPC4 transcript variant Zeta was obtained in the pTriEx-1.1 vector (mrgene) incorporating a C-terminal HA and His tag (shown in Figure 2.4). The CRBP-GST construct (in pGEX-4T-3,h Figure 2.5) was already available in the lab. For cloning and plasmid amplification, supercompetent *E. coli* strain XL1-Blue was used. Expression of full length human RAIG2, RAIG3 and TRPC4 were carried out in mammalian HEK293 cells (Invitrogen) and in the cell free systems MembraneMax™. The CRBP-GST fusion protein was expressed in the *E. coli* strain BL21-CodonPlus (DE3)-RP.

2.3 Construction of the RAIG2/pET-30a (+) vector

The RAIG2 coding sequence was obtained in Origene's pCMV6-XL4 vector shown in figure 1. This vector contained no epitope tags for purification and detection of the protein once expressed. The RAIG2 sequence was subcloned into pET-30a (+) (Novagen) for expression as a His-fusion protein. This vector was used because it contained a C-terminal His Tag and also a T7 promoter for cell free expression.

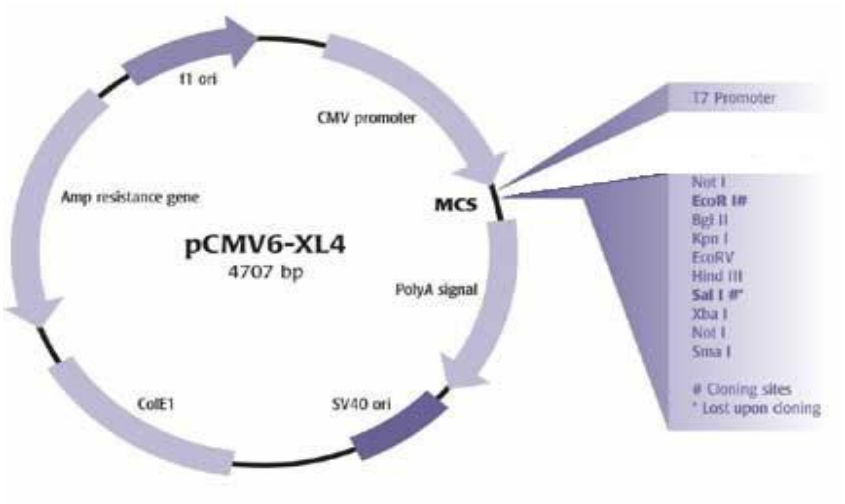


Figure 2.1: The pCMV6-XL4 Vector (Origene).

The coding sequence for RAIG2 was obtained in the untagged expression vector pCMV6-XL4 (OriGene). A Cytomegalovirus promoter drives gene expression while the T7 promoter allows for cell free expression. The vector contains no tags for detection and purification of the expressed protein. The ORF is 1212 in length. (Taken from the OriGene TrueORF^{TM} manual).

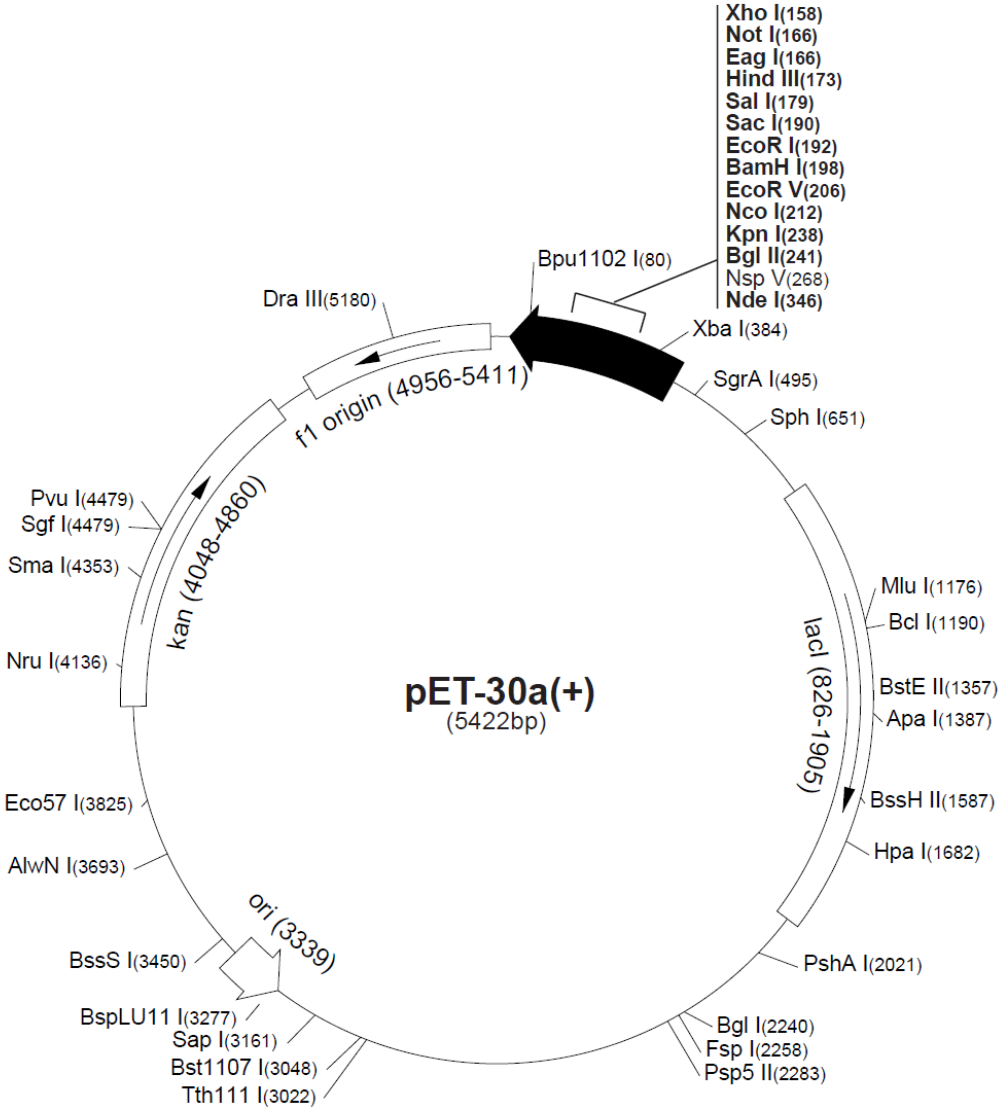


Figure 2.2: The pET-30a(+) Vector (Novagen).

The coding sequence for RAIG2 was cloned into the *E-coli* expression vector pET-30a(+). A T7 promoter drives gene expression. The vector contains His and S-tags for detection and purification of the expressed protein. The vector is 5422bp and through its T7 promoter allows for cell free expression. The RAIG2 sequence was ligated into the vector containing the Nde1 and Xho1 restriction sites on its N and C-terminal, to express the RAIG2 protein with a C-terminal His tag. (Taken from the Novagen pET system manual)

2.3.1 Transfecting E-coli cells with pCMV-XL4 vector

For cloning and plasmid amplification, supercompetent *E.coli* strains XL1-Blue were transformed. Approximately 25µl of competent XL-1 Blue cells (Stratagene) were transformed with 100ng of the RAIG2/pCMV construct. The transformation was incubated on ice for 30 minutes. Transformed cells were then heat shocked for 30 seconds at 42 ^{0}C and left on ice to recover for 2 minutes. Approximately 500µl of LB broth (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl) was added prior to incubation for 1 hour at 37^{0}C, shaking under 200rpm. Following the incubation period, resuspended cells (100µl) were plated on agar plates (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, 15g/L agar) with appropriate antibiotic (Ampicillin 50µg/ml) and allowed to incubate overnight at 37 ^{0}C, shaking at 200rpm. 4 colonies were then picked and allowed to incubate overnight at 37 ^{0}C, shaking at 200rpm, in 3mls LB ampicillin (50µg/ml). After the overnight incubation period, the plasmid DNA was extracted from the cells using Qiagen miniprep kits. DNA concentration values were obtained, taken at a 260/280 wavelength, using nanodrop spectrometry.

2.3.2 Plasmid DNA Purification

Plasmid DNA amplified in XL-1 Blue competent cells was purified using QIAprep Spin HiSpeed Miniprep and Plasmid Midi kits (Invitrogen) according to the manufacturer's instructions. Briefly, following alkaline lysis of bacterial cells denatured proteins, genomic DNA, and cell debris were cleared from the lysate by centrifugation (Miniprep) or filtration for larger volumes. To allow purification of plasmid DNA from small bacterial cell cultures, lysates were prepared under alkaline conditions, neutralised, and cleared of genomic DNA, denatured proteins, and cell debris by centrifugation. DNA was then adsorbed onto a silica membrane in high-salt buffer, washed, and subsequently eluted in a low salt buffer. Concentration and purity of final DNA preparation was estimated by agarose gel electrophoresis (2 (C)) and verified by spectroscopy. Successful constructs were independently sequenced (Eurofins) to verify the coding sequence was correct, in frame, and free of replication errors.

2.3.3 Agarose Gel Electrophoresis of DNA

Agarose gel electrophoresis was used to determine the correct size of PCR products, estimate concentration of DNA, confirm restriction digestion, and for verification of successful ligation of inserts into destination vectors. Dependent upon the size(s) of DNA to be visualised, 1-1.5% (w/v) agarose was dissolved in 1X TAE (40Mm Tris-acetate, Ph 8.0, 1 mM EDTA) by heating. The solution was allowed to cool prior to the addition of SYBR® Safe DNA gel stain (Invitrogen, 10,000X concentration in DMSO) to facilitate visualisation of DNA. Gels were subsequently poured and allowed to set at room temperature for one hour before use. Samples were prepared in a 5X DNA loading buffer supplied with the markers.

Agarose gels were electrophoresed in 1X TAE buffer at ca. 5 volts/cm and DNA visualised under UV light once sufficient migration and separation had occurred. Size markers (100bp and 1 Kbp DNA ladder, Promega) in addition to the quantitative marker HyperLadder^{TM} I (Bioline), were used for estimation of fragment length and DNA concentration, respectively.

2.3.4 The Polymerase Chain Reaction (PCR)

Subcloning of the coding sequence of RAIG2 was by amplification from the pCMV/RAIG2 vector by PCR. The coding sequence was amplified using the following primers:

Forward primer: $^{5'}$-CATATGTTCGTGGCATCAGAGAAAGATG-$^{3'}$

Reverse primer: $^{5'}$-CTCGAGCCAAAGGTGTCTTCCTGTG-$^{3'}$

The primers included recognition sequences for the restriction enzymes Nde1 ($^{5'}$….CATATG….$_{3'}$) AND Xho1 ($^{5'}$….CTCGAG….$_{3'}$) and the relevant part of the sequence. A 50µl PCR reaction was set up. DNA template (50ng) was combined with each of the primers (at a final concentration of 20µm), dNTPs (0.2mM each), and $MgSO_4$ (1.5mM), in a Pfu 10x reaction buffer (Agilent) to a final volume of 49µl. After thorough mixing, 1µl of Pfu Hot Start Polymerase (Stratagene) was added. Following polymerase activation at 95 ^{0}C for 5 minutes, PCR was performed for 30 cycles of denaturation at 95 ^{0}C for 30 seconds, annealing at 62 ^{0}C for 30 seconds, and extension at 72 ^{0}C for 1 minute and 30 seconds. Total reaction mixture was analysed on a 0.7 % (w/v) agarose gel. The gel was run at 100v for 35 minutes. Where a single PCR product of expected size was observed, the band was excised under U/V light and purified using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Briefly, the excised band was dissolved in buffer containing a high concentration of chaotropic salts allowing adsorption to a silica-gel membrane, impurities removed by washing with an ethanol based buffer, and DNA eluted in a double

distilled MilliQ water. DNA concentration values were obtained, taken at a 260/280 wavelengths.

2.3.5 TOPO ligation

The PCR product was then ligated into Invitrogen's Zero Blunt TOPO PCR Cloning Kit. Approximately 250ng of purified PCR product was added to 1µl PCR blunt II TOPO vector and 1µl salt solution, followed by an incubation period of 5 minutes at R/T. After the incubation period, 2µl of the TOPO reaction was added to a vial containing 250µl of One Shot TOP10 chemically competent *E. coli* cells (Invitrogen). The cells were transfected and mixed and incubated on ice for 30 minutes. The cells were then heat shocked at 42 ^{0}C for 30 seconds and placed on ice for 2 minutes to recover. Approximately 250ul of SOC medium was added to the cells prior to incubation at 37 ^{0}C for 1 hour, shaking at 200rpm. After the incubation period, 100ul of the resuspended cells were plated on 50µg/ml Kanamycin agar plate and left to incubate O/N at 37 ^{0}C. After the incubation period, 7 colonies were observed on the plate. These colonies were picked and each was placed in falcons containing 3mls LB media and kanamycin (50µg/ml). They were then left at 37 ^{0}C O/N, shaking at 200rpm. Cell growth was observed in only 1 of the falcons. Mini preps were performed on these cells to extract the TOPO vector from the cells. DNA concentration was calculated and a restriction digest was performed with the restriction enzymes Nde1 and Xho1. Approximately 52ng of TOPO vector was incubated with both enzymes at 10U/µl and BSA (100ng/ml) in a 1X reaction buffer (Promega buffer D, 6mM Tris-HCl, 6mM $MgCL_2$, 150mM $NaCl_2$, 1mM DTT, pH 7.9), for 3 hours at 37^{0}C. A 0.7% agarose gel was made up and 15µl of the digest was pipetted into the 2nd well. The 1st well contained 10µl of Fermentas 1kb DNA ladder.

The gel was run for 35 minutes at 100v. Larger quantities of DNA were extracted using Invitrogen Midi prep kits.

2.3.6 Ligation of RAIG2 into the pET vector

Both the TOPO vector and destination vector (pET) were linearised by double restriction digest, followed by ligation of the coding sequence into the pET vector. A double restriction digest was performed on the pET vector and the TOPO/RAIG2 vector using the enzymes Nde1 and Xho1 (Promega). Both digests were incubated with both enzymes at 10U/µl and BSA (100ng/ml) in a 1X reaction buffer (Promega buffer D, 6mM Tris-HCl, 6mM $MgCL_2$, 150mM $NaCl_2$, 1mM DTT, pH 7.9), for 3 hours at 37^0C. Total reaction volumes were then run in a 0.7% agarose gel at 100v for 35 minutes. The digested Pet vector and RAIG2 insert were gel purified and their DNA concentrations obtained. Approximately 100ng of pET vector and a 1-fold, 2-fold and 3-fold excess of insert were mixed in a 1X ligation buffer (NEB, 50mM Tris-HCl, 10mM MgCl2, 10mM DTT, 1mM ATP, pH 7.5), to which 1µl (2000U) Quick T4 DNA Ligase (NEB) was added to a final volume of 20µl. Total reaction volume mix was incubated at 16^0C O/N. Competent XL-1 Blue cells (stratagene) were transformed with 25ng of the ligation products. The transformed cells were then allowed to incubate for 30 minutes on ice. Cells were then heat shocked at 42 ^{O}C for 45 second before being returned to ice for a further 2 minutes. Pre-heated SOC media (900µl) was added prior to incubation at 37 ^{0}C, with shaking (200rpm), for 1 hour. Following the incubation period, 100µl of the transformation mixture were spread onto Kanamycin plates (100µg/ml), followed by an incubation period O/N AT 37 ^{0}C, shaking at 200rpm. After the incubation period colonies were observed on each of the plates.

1:1 Plate **2:1 Plate** **3:1 Plate**

10 colonies 7 colonies 8 colonies

5 colonies were scraped from each plate and placed into 3mls of LB/Kanamycin (50µg/ml). They were allowed to incubate at 37^{o}C, with shaking at 200rpm, O/N. After the incubation period, all the colonies grew in the media. Mini preps were performed on each colony and the DNA concentration was obtained. 20µl restriction digests were set up on each colony, using Nde1 and Xho1, to cut the double stranded DNA. A 0.7% agarose gel was made and each digest was loaded into the wells. The gel was run at 100v for 35 minutes.

2.4 RAIG3/pCMV6-Entry vector

Origene's RAIG3/pCMV6-Entry vector was used to express the RAIG3 protein. This vector contained a T7 promoter for cell free expression and also a CMV promoter for HEK cell expression. It also contained a C-terminal Myc and Flag Tag for protein detection and purification. The vector map is shown in figure 2. The vector was digested with BamH1 and Xho1 to confirm the RAIG3 insert.

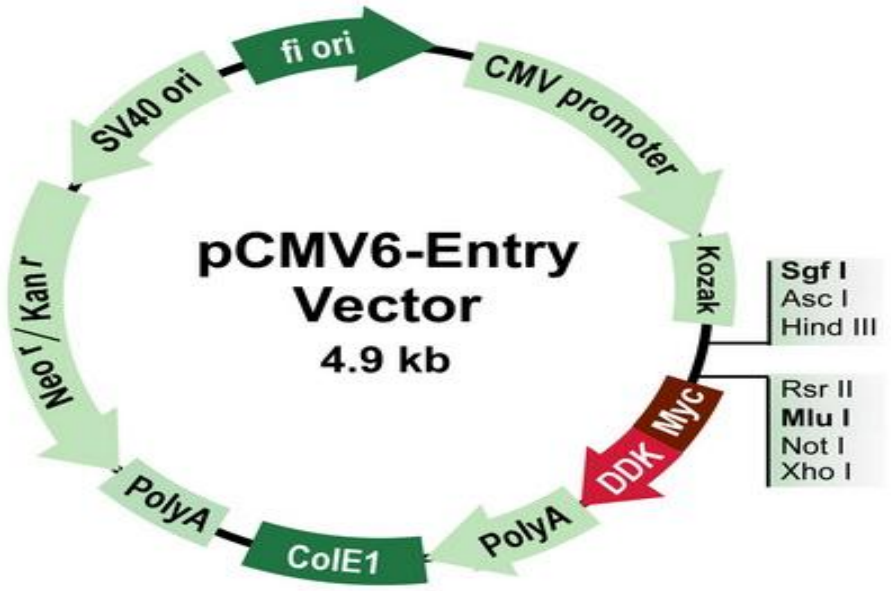


Figure 2.3: The pCMV6-Entry Vector (Origene).

The coding sequence for human RAIG3 was obtained in functional mammalian expression vector pCMV6-entry (Origene). The vector incorporates a C-terminal Myc and DDK-tag and a kozak consensus sequence. A Cytomegalovirus promoter drives gene expression while the T7 promoter allows for in vitro transcription/translation. The ORF is 1461 bp in length. (Taken from the Origene TrueORF^{TM} manual).

2.5 Cell Free Expression of RAIG2 and RAIG3

Invitrogen's MembraneMax™ Cell-Free Expression Systems was used to try and express RAIG2 and RAIG3. This expression system only requires 1µg of template DNA and promises microgram to milligram quantities of protein. The cell free reaction consisted of:

- E.coli slyD^{-} Extract 20µl
- 2.5X IVPS Reaction Buffer (-amino acids) 20µl
- 50mM Amino Acids (-Met) 1.25µl
- 75mM Methionine 1µl

- MembraneMax Reagent 2µl
- T7 Enzyme 1µl
- DNA Template (RAIG2/3) 1µg
- DNase/RNase-Free Water To a final volume of 50µl

The 1.5ml microcentifuge tube was closed and allowed to incubate at 37 ^{0}C, shaking at 200rpm, for 30 minutes. The reaction also consisted of a feed buffer which consisted of:

- 2X IVPS Feed Buffer 25µl
- 50mM Amino Acids (-Met) 1.25µl
- 75mM Methionine 1µl
- DNase/RNase-free Water to a final volume of 50µl

After the 30 minute incubation period, 50µl of this feed buffer was added to the reaction. The reaction was incubated at 37 ^{0}C, under 200rpm shaking, for 2 hours. The reaction was then stored at -20 ^{0}C.

2.5.1 SDS-PAGE

Separation of proteins according to molecular weight was achieved by SDS-PAGE to allow detection of expression. Approximately 50µl of each of the RAIG2 and RAG3 cell free

expression reaction were electrophoresed in 10% polyacrylamide gel. Samples were first prepared in loading buffer [50mm Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2 % (w/v) SDS, 100mM β-mercaptoethanol, Bromophenol blue] and incubated at 60 ^{0}C for 10 minutes prior to loading onto gels. Electrophoresis was carried out in 1X Tris-HEPES tank buffer (0.1 M Tris, 0.1 M HEPES, 3 mM SDS, pH 8) for 1 hour 30 minutes at 100v.

2.5.2 Western Blotting to detect expression of RAIG2 and RAIG3

The 2 SDS-PAGE gels, containing the proteins of interest, were transferred onto a PDVF membrane in transfer buffer [10mM CAPS, pH11, 10% (v/v) methanol] for 2 hours at 140mA, using a semi-dry transfer unit. Membranes were then blocked in 10 % (w/v) non-fat dry milk (NFDM) in PBS-t [10mM NaH_2PO_4, PH 7.4, 0.14M NaCl, 3mM KCl. 0.05 % (v/v) Tween-20], 0/N at 4 ^{0}C. Membranes were then washed in PBS-t (5 x 5 minutes).

For detection of the proteins, the membrane containing the RAIG2 sample was incubated with a 1:10,000 dilution of the His-HRP conjugated antibody [in PBS-t, 1 % (w/v) NFDM] for 2 hours at R/T, then washed in PBS-t (5 x 5 minutes). The membrane containing the RAIG3 sample was incubated with a 1:1000 dilution of the Flag antibody (Origene) for 2 hours at R/T, washed in PBS-t (3 x 5 minutes), then incubated with a 1:5000 dilution of the anti-mouse IgG HRP-Conjugated secondary antibody [in PBS-t] for 1 hour and finally washed in PBS-t (5 x 5 minutes). After the washing steps, the membranes were developed by incubation for 2 minutes in ECL Western blotting substrate (Pierce), and signal detected by X-ray film exposure using G.R.I Blue sensitive film (Kodak) and a Xograph Compact X4 film processor.

2.6 Protein expression in HEK293T™ cells

For the purpose of protein expression, HEK293T™ Cell Lines were to be transfected. The RAIG3 and TRPC4 coding sequences were in vectors that allowed for mammalian expression. The RAIG3 sequence was in the pcmv-Entry vector with a CMV promoter which allowed for gene expression in HEK293 cells. The TRPC4 sequence was in the pTriEx1.1 vector, with a CMV immediate early enhancer fused to the chicken β-actin promoter, which allowed for gene expression in HEK293 cells. The RAIG2 sequence needed to be subcloned into a mammalian expression vector, as it was in a vector that did not allow mammalian expression (pET vector).

2.6.1 Subcloning RAIG2 into the pCMV-Entry vector.

In order to express RAIG2 in HEK cells, the sequence had to be subcloned into a mammalian expression vector. The mammalian vector chosen was the pCMV-Entry vector. The coding sequence of RAIG2 was amplified from the pet vector by PCR, using the primers, $^{5'}$-GCGATCGCCATGTTCGTG-$_{3'}$ and $^{5'}$-CTCGAGCCAAAGGTGTCTTCCTGTG-$_{3'}$. Primers included recognition sequences for the restriction enzymes Sgf1 ($^{5'}$….GCGATCGC….$_{3'}$) and Xho1 ($^{5'}$….CTCGAG….$_{3'}$) respectively, and the relevant part of the RAIG2 coding sequence. DNA template (50ng) was combined with each of the primers (at a final concentration of 20µm), dNTPs (0.2mM each), and $MgSO_4$ (1.5mM), in a Pfu 10x reaction buffer (Agilent) to a final volume of 49µl. After thorough mixing, 1µl of Pfu Hot Start Polymerase (Stratagene) was added. Following polymerase activation at 95 ^{0}C for 5 minutes, PCR was performed for 30 cycles of denaturation at 95 ^{0}C for 30 seconds, annealing at 64 ^{0}C for 30 seconds, and

extension at 72 ^{0}C for 2 minutes. Total reaction mixture was analysed on a 0.7 % (w/v) agarose gel. The gel was run at 100v for 35 minutes. Where a single PCR product of expected size was observed, the band was excised under U/V light and purified using the QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions.

2.6.2 Ligation of RAIG2 into the pCMV-entry vector

The purified PCR product was digested with the restriction enzymes Sgf1 and Xho1. Total PCR product and destination vector (pCMV-Entry) were incubated with both enzymes at 10U/µl and BSA (100ng/ml) in a 1x reaction buffer (Promega buffer C, 10mM Tris-HCl, 10mM $MgCL_2$, 50mM $NaCl_2$, 1mM DTT, pH 7.9) for 3 hours at 37^{0}C. After the incubation period, total volume of digested vector and insert were run on a 0.7 % agarose gel and purified as described before. After the purification, DNA concentration was quantified using nanodrop spectrometry. Approximately 32ng of vector and a 3-fold molar excess of insert were mixed in a 1x ligation buffer (NEB, 50mM Tris-HCl, 10mM MgCl2, 10mM DTT, 1mM ATP, pH 7.5), to which 1µl (2000U) Quick T4 DNA Ligase (NEB) was added to a final volume of 20µl. The total reaction mixture was incubated at 16 ^{0}C O/N. Competent XL-1 Blue cells (Stratagene) were then transformed with 20ng of the ligation product. The transformed cells were allowed to incubate for 30 minutes on ice. Cells were then heat shocked at 42 ^{O}C for 45 second before being returned to ice for a further 2 minutes. Pre-heated SOC media (900µl) was added prior to incubation at 37 ^{0}C, with shaking (200rpm), for 1 hour. Following the incubation period, 100µl of the transformation mixture were spread onto Kanamycin plates (100µg/ml), followed by an incubation period O/N at 37 ^{0}C. After the incubation period colonies were observed on the plate.

2.6.3 HEK Cell Transfection

FuGENE® HD (Roche) was the transfection reagent used to transfect HEK293T cells with my RAIG3/pCMV DNA template. To 60µl OPTI-MEM® I media, 9µl of Fugene was added, followed by an incubation of 5 minutes. Following the incubation period, approximately 8.4µg of template was added, followed by a 20 minute incubation period after which, approximately 5.6 x 10^5 HEK cells were pelleted by centrifugation (5,000g) at 4^0C for 10 minutes. The supernatant was discarded and the resultant pellet resuspended in 1ml of OPTI-MEM® I media. Suspended cells were added to 4 wells of a 6 well flat bottom plate (Sarstedt). The transfection mix was then added drop wise to the cells and incubated for 48 hours at 37^0C. Following the incubation period, cells were washed in ice cold 10x PBS (2 X 1ml). The cells that adhered to the surface were then scraped off, using a cell scraper (Sarstedt), into 1ml of ice cold 1x PBS and pelleted by centrifugation (5000g) at 4^0C for 10 minutes. The resultant pellet was lysed in 300ul lysis buffer (PBS, 1 % DDM) with rolling at 4^0C and the debris pelleted by centrifugation (13,000g) for 15 minutes at 4^0C. Approximately 30µl of the supernatant was analysed SDS-PAGE and Western blotting. The RAIG2/pCMV vector was used for expression and analysis using this same protocol.

2.6.4 Membrane Preparation of RAIG3

FuGENE® HD (Roche) was the transfection reagent used to transfect HEK293T cells with my RAIG3/pCMV DNA template for the membrane prep. To 600µl OPTI-MEM® I media, 90µl of Fugene was added, followed by an incubation of 5 minutes. Following the incubation period, approximately 84µg of template DNA was added, followed by a 20 minute incubation period after which, approximately 5.6 x 10^6 HEK cells were pelleted by centrifugation (5,000g) at 4^0C for 10 minutes. The supernatant was discarded and the resultant pellet resuspended in 10ml of OPTI-MEM® I media and added to a 175cm^3 flask. The transfection mix was then added drop wise to the cells and incubated for 48 hours at 37^0C. Following the incubation period, cells were washed in ice cold 10x PBS (2 X 10ml), the adherent cells scraped off using a cell scraper (Sarstedt), into 10ml of ice cold 1x PBS and pelleted by centrifugation (1000g) at 4^0C for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 2ml hypotonic buffer (10mM HEPES, pH 7.9, 1.5mM $MgCl^2$, 10mM KCl, 0.5mM DTT, Protease inhibitors (Sigma) and ruptured by freeze/thawing, 5 times in a dry ice/EtOH bath and in a 37^0C water bath. The cells were then sonicated for 1 minute at 10 % on ice and the cell debris pelleted using centrifugation (3000g) for 15 minutes at 4^0C. The supernatant was retained and made up to 5ml in hypotonic buffer. To this 18µl of $CaCl_2$ was added, followed by vortexing for 5 minutes. The sample was then loaded into 5ml pollyallomer Optiseal tubes (Beckman), and the membranes pelleted using centrifugation (100,000g) for 1 hour at 4^0C. The supernatant was discarded and the membranes were resuspended in 50µl MES buffer (1 % triton (v/v), 25mm Mes, 0.15m NaCl, protease inhibitors, pH 6.5), using a 25G needle and a syringe. The sample was left on ice and at 15 minute intervals, the membrane granules were resuspended. Protein was quantified using a BSA protein assay, followed by analyses by SDS-PAGE and Western blotting.

2.6.5 TRPC4 Zeta/pTriEx-1.1 Construct

The TRPC4 Zeta construct was obtained from mrgene.com (Figure 3). The coding sequence for TPRC4 Zeta was constructed to incorporate a C-terminal HA and His tag. This vector was used to express the full length ion channel in HEK293 cells. Transient expression is mediated by a hybrid promoter composed of the CMV immediate early enhancer fused to the chicken β-actin promoter.

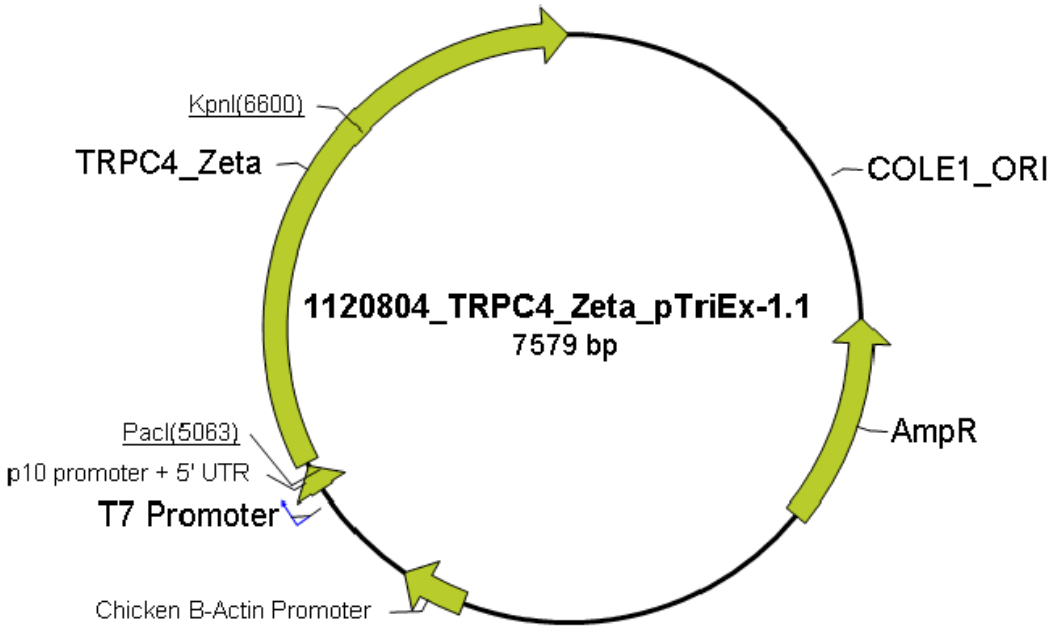


Figure 2.4: The pTriEx-1.1 Vector (mrgene).

The coding sequence for TPRC4 Zeta was obtained in the mammalian expression vector pTriEx-1.1 (mrgene). A hybrid promoter composed of the CMV immediate early enhancer fused to the chicken β-actin promoter drives gene expression while the T7 promoter allows for in vitro transcription/translation. The vector contains C-terminal HA and His tags for detection and purification of the expressed fusion protein. The ORF is 1212 in length. (Taken from mrgene.com website).

2.6.6 TRPC4 Expression in HEK293T Cells

HEK293T cells were used to express the TRPC4 fusion protein. Approximately 2 x 10^5 cells in Hyclone DMEM media (1 % L-glutamine, 1 % Penicillin Streptomycin, 10 % Foetal Calf Serum), were plated in a well of a 6 well plate, and incubated at 37^0C for 24 hours. Template (TRPC4, 2ug) was diluted in 250µl of OPTI-MEM® 1 media and incubated for 5 minutes at R/T. Lipofectamine® 2000 reagent (Invitrogen,5µl) was diluted in 245µl of OPI-MEM® 1 media and incubated at 37^0C for 5 minutes at R/T. Following the incubation period, both samples were mixed and left to incubate for 20 minutes at R/T after which, the transfection mix was added drop wise to the cells in the 6 well plate. The cells were left to incubate for 24 hours at 37^0C, cells then washed in ice cold 10x PBS (2 X 1ml) and the adherent layer scraped off using a cell scraper (Sarstedt), into 1ml of ice cold 1x PBS. The cells were then pelleted by centrifugation (5000g) at 4^0C for 10 minutes. The supernatant was discarded and the resultant pellet was lysed in 300ul lysis buffer (50mM HEPES pH 7.5, 1mM EDTA, 10% glycerol, 0.05% Chaps, 1% Triton X, 150mM NaCl) with rolling at 4^0C, for 2 hours. Following the incubation period, the mixture was pelleted by centrifugation (13,000g) for 15 minutes at 4^0C and 30µl of the supernatant was analysed SDS-PAGE and Western blotting.

2.7 CRBP Expression in *E.coli*

The coding sequence of CRBP incorporating a C-terminal GST tag was obtained in the vector pGEX-4T-3 (Invitrogen),. The vector was used to express a CRBP-GST fusion protein in bacteria driven by the T7 promoter. For the purpose of protein expression, competent cells from the *E. coli* host strain BL21 Gold (Invitrogen) were transformed. The cells were spread on LB-Ampicillin (50µg/ml) plates and incubated O/N at 37^0C. After the incubation period, 50mls of LB-Ampicillin (50µg/ml) media was inoculated with a colony from the O/N plates

and incubated O/N, shaking at 37^0C. 10ml of this pre-culture was inoculated in 1l of 2YT (Trytone 16g/L, Yeast extract 10g/L, NaCl 5g/L) with Ampicillin (50µg/ml) and incubated for 3 hours, shaking at 37^0C, until the optical density at 600nm (OD_{600}) of the culture was 0.4-0.6. The remaining 40ml was discarded. Protein expression was then induced by the addition of IPTG to a final concentration of 1mM and growth continued for a further 4 hours prior to harvesting the cells by centrifugation (5000rpm) at 4^0C for 15 minutes. The supernatant was discarded and resultant pellet resuspended in an appropriate volume of ice-cold PBS (3ml PBS per gram of pellet) supplemented with protease inhibitors (Complete, EDTA-free Protease Inhibitor Cocktail, Roche). The cells were lysed by the addition of 10mg of lysozyme and incubated for 30 minutes, at 37^0C with shaking. Cells were sonicated over ice in short bursts until the lysate cleared. DNase was then added to a final concentration of 4µg/ml, while incubation continued at 37^0C for 20 minutes, before centrifugation (12,000rpm) at 4^0C for 20 minutes to remove the insoluble fraction. The supernatant was retained and the pellet discarded.

Glutathione-Sepharose beads were washed in PBS (500µl beads per 25ml of bacterial lysate) and added to bacterial supernatant in a 50ml falcon tube on 4^0C rotator O/N. Following the incubation period, the resin was packed in a disposable 5ml column (Thermo scientific), retained and stored at 4^0C. The resin was washed (3 x 2ml) with 1X PBS. Elution buffer (50mM Tris-HCl, 10mM glutathione, pH8) was added (500µl resin: 1ml elution buffer) and sample was eluted (3 x 1ml) from beads into 15ml falcon tubes. Spectra/Por® Mini dialysis units (SpectrumLabs.com) were used to remove reduced glutathione into PBS at 4^0C O/N with stirring. Samples were run on a 12 % SDS-PAGE gel and visualised by staining in Coomassie brilliant blue [0.1 % (w/v) Coomassie brilliant Blue, 40 % (v/v) methanol, 10 % (v/v) acetic acid] for a minimum of 2 hours, prior to destaining with water.

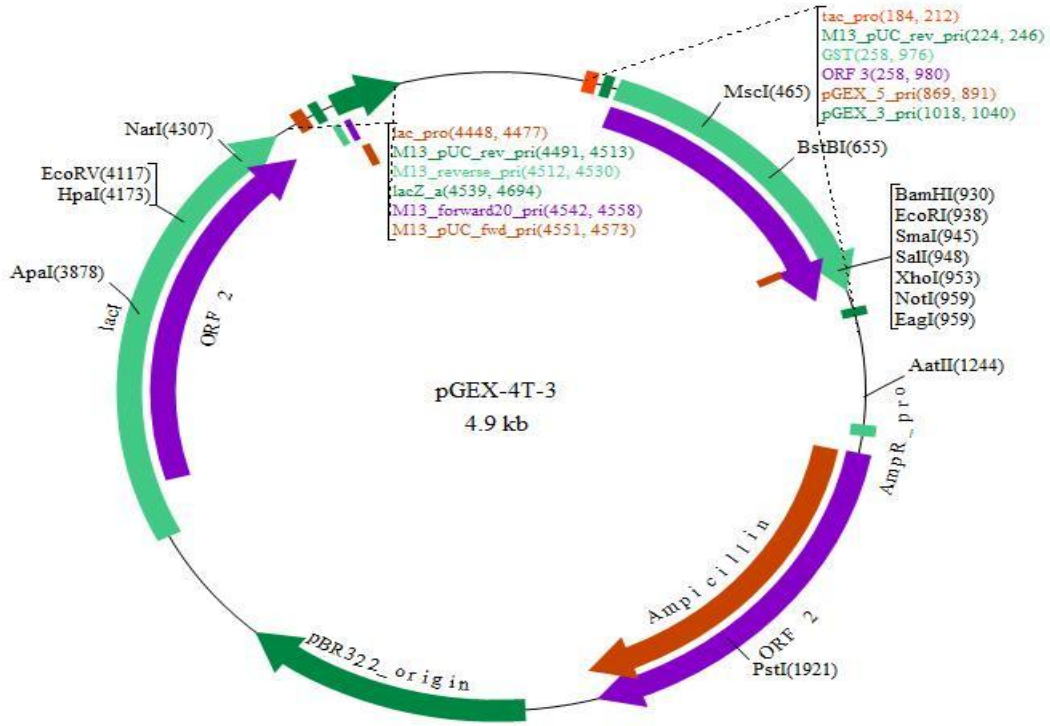


Figure 2.5: The GST Gene Fusion System Vector (pGEX-4T-3).

The pGEX-4T-3 vector containing the coding sequence for CRBP. The open reading frame also includes a sequence for a GST tag. *E. coli* expression of inserts cloned into pGEX-4T-3 yields N-terminally tagged GST fusion proteins. Gene expression is under the control of a *tac* promoter, inducible by the addition of IPTG. (Taken from the GST Gene Fusion System Handbook, Amersham Biosciences).

2.8 Protein interaction Studies

Protein: protein interactions were probed using a pull-down assay adapted to accommodate the possible low affinity of RBP for RAIG2 or RAIG3. A traditional wash step would potentially disrupt the receptor: ligand complex, and was therefore eliminated and substituted with centrifugation through a non-aqueous oil layer to minimize loss of any observable receptor: ligand complex through dissociation. Variations of this method have been used successfully in previous receptor:ligand binding assays (Segal & Hurwitz, 1977; Sivaprasadarao *et al*., 1988; Kita *et al*., 2009).

2.8.1 Oil Pull-down with RAIG2 and RBP

To 1µg of RBP, 0.95µg of retinol was added and allowed to incubate for 15 minutes at R/T. Approximately 50µl of soluble RAIG2 was immobilised on an anti c-myc agarose affinity gel (50µl) O/N at 4^0C. After the incubation period, 100ng of RBP/retinol was added and incubated for 2 hours, rolling at 4^0C. Components were resuspended and carefully layered on top of an oil mix (3:2 mix dibutyl phthalate: dinonyl phthalate, both Sigma, pre-warmed to 37 ^{0}C). Centrifugation was carried out at 15,000 rpm in a bench top centrifuge at R/T for 20 minutes until 3 distinct layers were visible. Aqueous and oil layers were then carefully removed and the oil layer discarded. The sedimented layer containing anti c-myc agarose affinity gel, bound domain and any interacting partners, was incubated with 2X SDS sample buffer for 10 minutes at 60^0C. The aqueous layer was also saved, and incubated in 2X SDS sample buffer for 10 minutes at 60^0C. The resin was then sedimented by centrifugation (13,000 rpm, 1 minute) and the supernatant removed and analysed by SDS-PAGE and Western blotting. The aqueous layer was also analysed by SDS-PAGE and Western blotting.

2.8.2 Stripping and Reprobing PVDF Membranes.

In order to visualise two bands on the same membrane, PVDF membranes were stripped using Restore^{TM} Plus Western Blot Stripping Buffer, and reprobed. Membranes were washed in PBS-t to remove ECL, prior to incubation with Restore^{TM} Plus Western Blot Stripping Buffer for 8 minutes at room temperture. Following incubation membranes were washed in PBS-t and subsequently blocked [10 % (w/v) NFDM] for one hour at room temperature or overnight at 4 ^{0}C. In order to ascertain complete removal of any secondary antibody used for initial detection, membranes were washed (3 x 5 minutes PBS-t) and developed as described previously. The stripping proccess was crucial as each of the pull down blots were stripped and reprobed. Generally the least efficient antibody was used first followed by stripping and reprobing with the highly efficient antibody.

2.8.3 Oil Pull-down with RAIG3 and RBP

To 1µg of RBP, 0.95µg of retinol was added and allowed to incubate for 15 minutes at R/T. Approximately 5µg of soluble RAIG3 membranes was immobilised on an anti c-myc agarose affinity gel (50µl) O/N at 4 ^{0}C. After the incubation period, 100ng of RBP/retinol was added and incubated for 2 hours, rolling at 4 ^{0}C. Following incubation components were

resuspended and carefully layered on top of an oil mix (3:2 mix dibutyl phthalate: dinonyl phthalate, both Sigma, pre-warmed to 37 ^{0}C). Centrifugation was carried out at 15,000 rpm in a bench top centrifuge at R/T for 20 minutes until 3 distinct layers were visible. Aqueous and oil layers were then carefully removed and the oil layer discarded. The sedimented layer containing anti c-myc agarose affinity gel, bound domain and any interacting partners, was incubated with 2X SDS sample buffer for 10 minutes at 60 ^{0}C. The aqueous layer was also saved, and incubated in 2X SDS sample buffer for 10 minutes at 60 ^{0}C. Following the incubation period the resin was sedimented by centrifugation (13,000 rpm, 1 minute) and the supernatant removed and analysed by SDS-PAGE and Western blot, as described previously. The aqueous layer was also analysed by SDS-PAGE and Western blot.

2.8.4 Standard Pull-down with CRBP and TRPC4

Protein: protein interactions were probed using a traditional wash step followed by elution of a possible receptor: ligand complex. Approximately 0.95µg of retinol was added to 1µg of CRBP-GST and incubated at R/T for 15 minutes. After the incubation period, 100ng of CRBP-GST/retinol was added to 30µl of Glutathione-Sepharose beads (GE Healthcare) and incubated, rolling at 4 ^{0}C, O/N. The Beads were then washed with PBS (1 X 500µl). Solublilised TRPC4 Zeta (100µl) was added to CRBP-treated-resin. In conjunction with this, 100µl of soluble TRPC4 Zeta was added to 30µl of untreated Glutathione-Sepharose beads as a negative control. Both reactions were allowed to incubate at 4^{0}C for 3 hours. The resin beads were then spun down by centrifugation (13,000g) at 4^{0}C for 1 minute. The supernatants were collected, the resins washed (3 x 100µl) with 1x PBS (+ protease inhibitors) and spun down by centrifugation (13,000g), after each wash, at 4^{0}C for 1 minute. After each wash, the supernatants were collected. After washing, the resins, were eluted (3 x 60ul) with elution

buffer (50mm Tris-HCl, 10mm glutathione, pH8), followed by an incubation period after each elution of 10 minutes at R/T. Following each incubation period, the resins were spun down through centrifugation (13,000g) for 1 minute at 4^0C and the supernatants collected. Each wash and elution was heated in 2X SDS-PAGE sample buffer at 60^0C for 10 minutes. The sedimented layer which contained the GST resin, bound domains and any interacting partners was also heated in sample buffer. The samples were analysed by SDS-PAGE and Western blotting, as described previously.

2.8.5 Oil pull-down with CRBP and TRPC4

To 1µg of cRBP, 0.95µg of retinol was added and incubated for 15 minutes at R/T. Approximately 100µl of soluble TRPC4 Zeta was immobilised on an Monoclonal Anti-HA-agarose (Sigma) (30µl) resin O/N at 4 ^{0}C. After the incubation period, 100ng of CRBP/retinol was added and incubated for 2 hours, rolling at 4 ^{0}C. CRBP was added to 30µl of Anti-HA agarose and incubated for 2 hours at 4^0C, as a negative control. Following incubation, components were resuspended and carefully layered on top of an oil mix (3:2 mix dibutyl phthalate: dinonyl phthalate, both Sigma, pre-warmed to 37 ^{0}C). Centrifugation was carried out at 15,000 rpm in a bench top centrifuge at R/T for 20 minutes until 3 distinct layers were visible. Aqueous and oil layers were then carefully removed and the oil layer discarded. The sedimented layer containing the Anti-HA agarose, bound domain and any interacting partners, was incubated with 2X SDS sample buffer for 10 minutes at 60 ^{0}C. The aqueous layer was also saved, and incubated in 2X SDS sample buffer for 10 minutes at 60 ^{0}C. Following the incubation period the resins were sedimented by centrifugation (13,000 rpm, 1

minute), the supernatants removed and analysed by SDS-PAGE and Western blotting. The aqueous layer was also analysed by SDS-PAGE and Western blotting.

Chapter 3

Cell Free Expression of RAIG2 and RAIG3

3.1 Introduction

This section describes the preparation, sub-cloning and expression of the RAIG2 and RAIG3 gene constructs. The section describes how the RAIG2 sequence was amplified by PCR and subcloned into the pET-30a vector, which contains a T7 promoter for Cell free expression. The RAIG3 sequence did not require subcloning, as the sequence was already in an expression vector with a T7 promoter (pCMV vector). This section also describes the expression of these gene constructs using the MembraneMax^{TM} cell free expression system.

3.1.1 Polymerase Chain Reaction

RAIG2 was successfully amplified containing Nde1 and Xho1 restriction enzyme recognition sequences. Total PCR product (50µl) was run on the gel. Strong bands appeared next to the DNA molecular marker for 1200bp. The RAIG2 coding sequence is 1212bp so this indicated that the PCR was successful and the desired RAIG2 sequence was amplified. This was verified by sequencing result. The primers used were as follows:

Forward primer: $^{5'}$-CATATGTTCGTGGCATCAGAGAAAGATG-$^{3'}$

Reverse primer: $^{5'}$-CTCGAGCCAAAGGTGTCTTCCTGTG-$^{3'}$

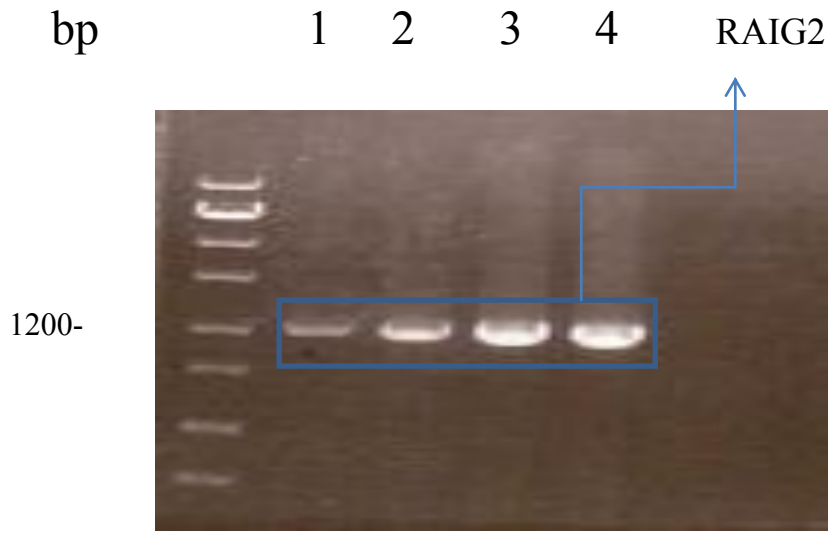


Figure 3.1: PCR Amplification of RAIG2 Sequence

PCR bands (lanes1-4) appear on the same line as 1200bp DNA marker. Lanes 1-4 contain 5, 10, 15, 20µl respectively of the PCR product.

3.1.2 TOPO Ligation

RAIG2 was successfully ligated into the TOPO vector. The resultant plasmid was cut with the restriction enzymes Nde1 and Xho1 and 200ng of the TOPO ligation product was run on a 0.7 % agarose gel. One band appeared above the 1000bp molecular marker corresponding to the insert (RAIG2 1212bp), which was successfully cut out of the TOPO vector. The other band appeared just under the 4000bp molecular marker which indicates the TOPO vector (3956bp) linearized.

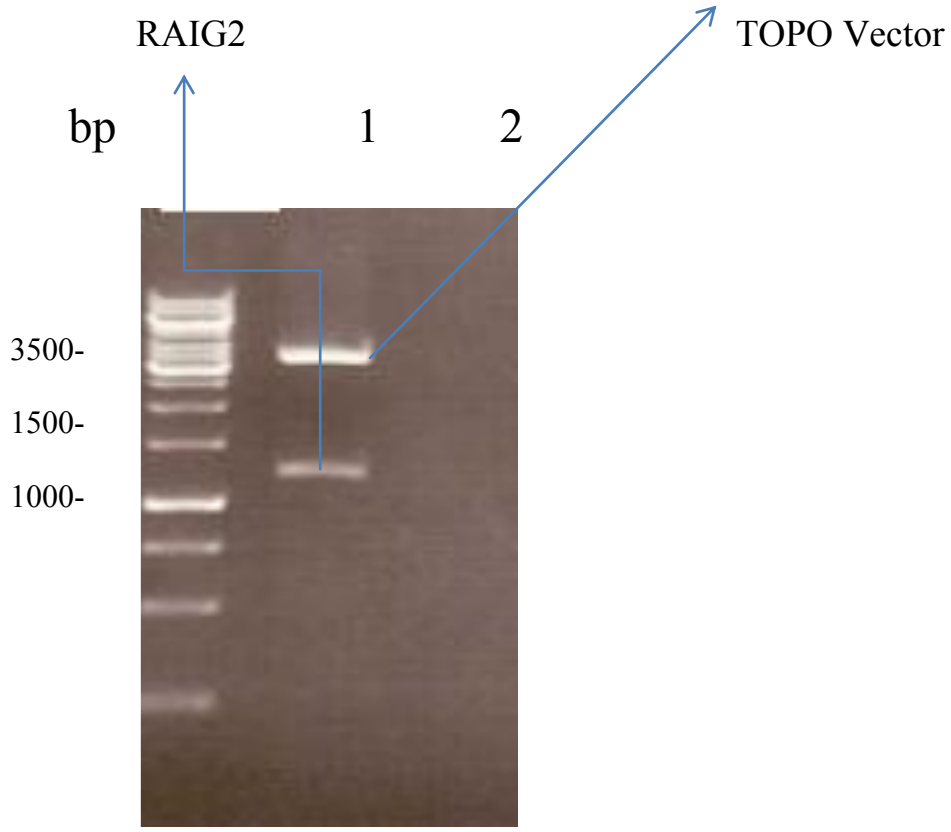


Figure 3.2: Ligation of RAIG2 Sequence into the TOPO Vector

TOPO/RAIG2 vector digested with Nde1 and Xho1 and run on a 0.7 % agarose gel. Correct bands are observed, one at 1200bp (RAIG2) and one at 3956bp (linearized TOPO vector). 200ng of DNA loaded into lane 1.

3.1.3 Ligation of RAIG2 into the pET vector.

Of the 15 colonies that grew on selective agar plates, the DNA was extracted, digested with Nde1 and Xho1 and run on a 0.7 % gel. Only one of the colonies contained the RAIG2 insert

which was correctly observed at 1212bp. The pET vector was observed at just over the 5000bp molecular marker (pET vector 5422bp) meaning it was linearized. This indicates that the RAIG2 coding sequence was successfully ligated into the pet vector using the restriction sites Nde1 and Xho1. This was verified by sequencing results. The vector was now ready for cell free expression of the RAIG2 protein.

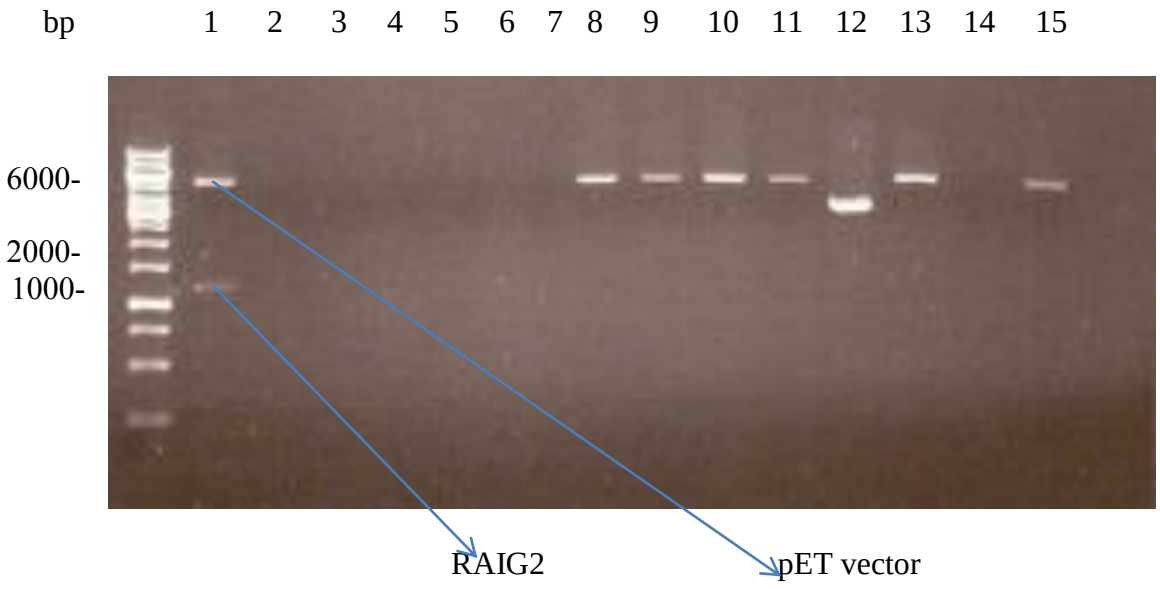


Figure 3.3: Ligation of RAIG2 into pET Vector.

Plasmid characterisation from colonies. Lane 1 contains the only bacterial colony that contained the ligated RAIG2/pET vector. The RAIG2 insert was observed correctly at 1212bp as was the linearized pet vector at 5422bp. 50ng of DNA was loaded into each well.

3.2 RAIG3/pCMV-Entry Vector

Origene's RAIG3/pCMV-Entry vector was digested with Sgf1 and Xho1. Bands were observed in lanes 1-3 just below 1500bp (RAIG3 1461bp) and just below 5000bp (pCMV vector 4900bp). This confirms that the RAIG3 coding sequence is in the pCMV vector flanked with the restriction sites, Sgf1 and Xho1. The vector was also successfully linearized using the restriction enzyme Xho1, indicated by a band above 6000bp in lanes 4-6 (Vector with insert 6361bp).

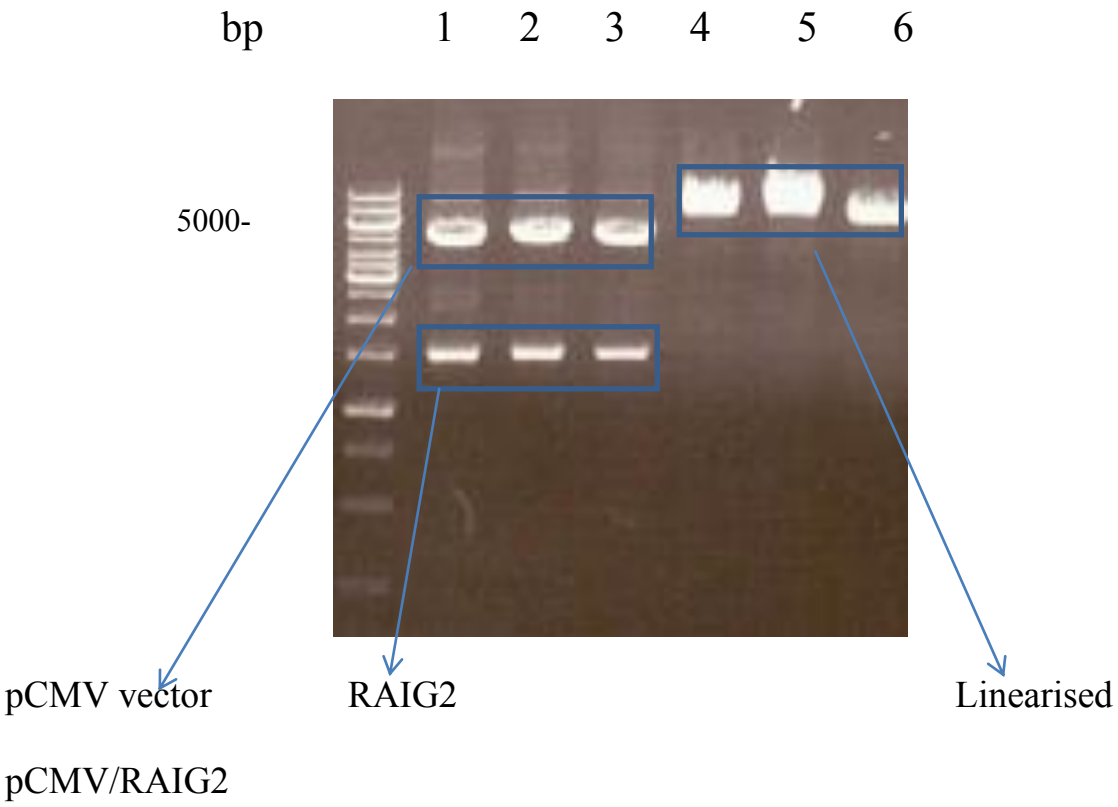


Figure 3.4: RAIG3/pCMV Restriction Digest.

RAIG3/pCMV Digested with Sgf1 and Xho1 (Lanes 1-3). RAIG3 Coding sequence (1461bp) and pCMV vector (4900bp) observed at the right size. RAIG3/pCMV vector successfully digested with Xho1 with a band observed at 6361bp (Lanes 4-6). 200ng of DNA loaded into each well.

3.3 Cell free expression of RAIG2 and RAIG3

The *E. coli* based MembraneMax™ expression system was used to express the RAIG2 and RAIG3 gene constructs. The system contains a T7 promoter which drives transcription of the

protein. It allows for quick and easy expression of proteins with the total reaction time lasting less than 4 hours. This is a great advantage over other cell free systems which take days to express proteins. It also produces micrograms of soluble membrane protein, which eliminates the difficult process of solubilising proteins.

3.3.1 Western blot of RAIG2

To look for RAIG2 protein expression in the cell-free assay, the His antibody (1:10,000 dilution) was used to detect the RAIG2-His fusion product. As a positive control, 100ng of the RBP-His fusion protein was loaded into lane 3 of the SDS-PAGE gel. 50µl (half of the cell free reaction product) was loaded into Lane 1. The blot indicates that there was no cell free expression of RAIG2. The blot picked up RBP at 21kDa in lane 3, while Lane 1 containing the cell free expression, was blank. This indicates that the cell free expression system was not able to express full length RAIG2.

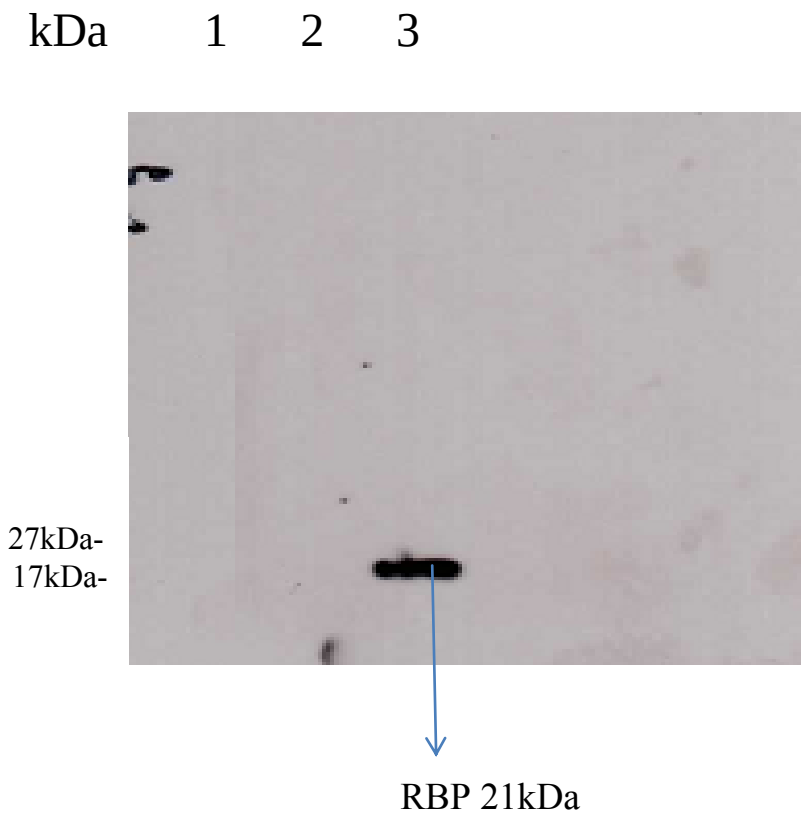


Figure 3.5: Western Blotting of Cell Free Expression of RAIG2.

50µl of cell free reaction product (Lane 1). 100ng His-RBP (Lane 3). All other lanes are blank. His antibody did not pick up any RAIG2 expression. His-RBP was detected as a positive control at 21kDa**.**

3.3.2 Western blot of RAIG3

To look for RAIG3 protein expression from the cell free assay, the c-Myc antibody (1:5000 dilution) was used in order to detect the RAIG3-c-Myc-DDK fusion product. As a positive control 100ng of Perforin (c-Myc tagged) was loaded into lane 1 of the SDS-PAGE gel. 50µl (half of the cell free reaction product) was loaded into Lane 3. The blot indicates that there was no cell free expression of RAIG3. The blot picked up Perforin at 42kDa in lane 1, while Lane 3 containing the cell free expression product, was blank. This indicates that the cell free expression system was not able to express RAIG3.

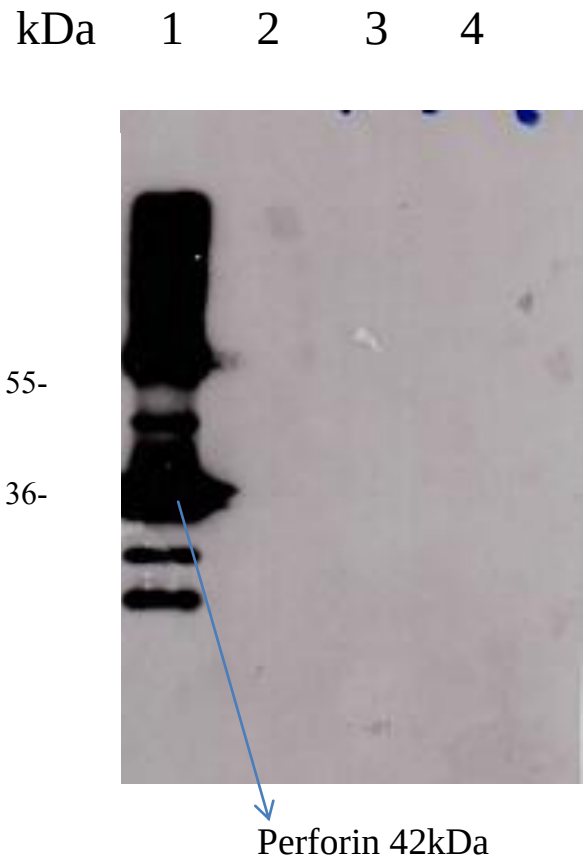


Figure 3.6: Western Blotting of Cell Free Expression of RAIG3.

100ng of Perforin-c-Myc (Lane 1). 50µl of cell free reaction product (Lane 3). All other lanes are blank. No RAIG3 expression was detected with c-Myc antibody. Perforin was detected at 42kd as a positive control in lane 1.

Chapter 4

RAIG2, RAIG3 and TRPC4 expression in HEK293 cells

4.1 Introduction

This section describes the preparation, sub-cloning and mammalian expression of RAIG2, RAIG3 and TRPC4. The section describes how the RAIG2 sequence was amplified by PCR and subcloned into the pCMV vector, containing a CMV promoter for mammalian expression. The RAIG3 and TRPC4 sequences did not require subcloning, as the sequences were already in an expression vector with a CMV promoter. This section also describes the expression of these gene constructs in HEK cells.

4.1.1 Polymerase Chain Reaction

RAIG2 was successfully amplified containing Sgf1 and Xho1 restriction enzyme recognition sequences. Total PCR product (100µl) was run on the gel. Strong bands appeared above the DNA molecular marker for 1000bp. The RAIG2 coding sequence is 1212bp so this indicated that the PCR was successful and the desired RAIG2 sequence was amplified. This was verified by sequencing results.

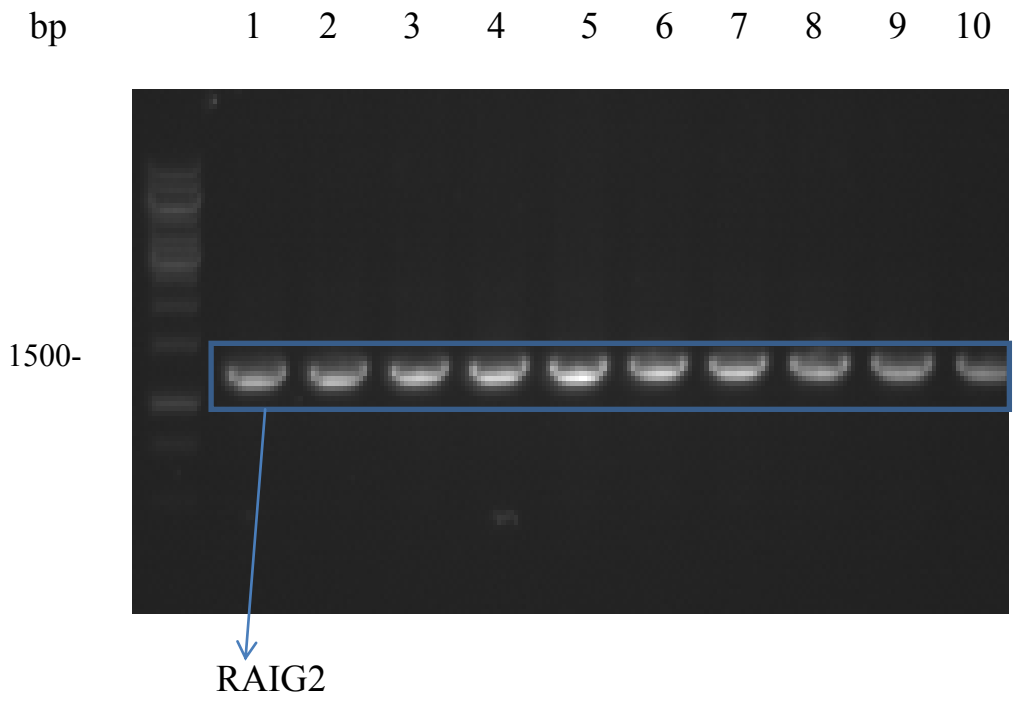


Figure 4.1: Amplification of RAIG2 by PCR.

10µl of PCR product was loaded into each lane. Band appears below 1500bp. RAIG2 coding sequence is 1212bp.

4.1.2 Ligation of RAIG2 into pCMV-Entry vector

The plasmid DNA extracted from the 3 colonies that grew on selective agar plates was digested with Sgf1 and Xho1 and run on a 0.7 % gel. All of the colonies contained the RAIG2 insert which was correctly observed at 1212bp. The pCMV vector was observed just under the 5000bp molecular maker (pCMV vector 4900bp) meaning it was linearized. This indicates that the RAIG2 coding sequence was successfully ligated into the pCMV vector containing the restriction enzyme recognition sites Sgf1 and Xho1. This was verified by sequencing results.

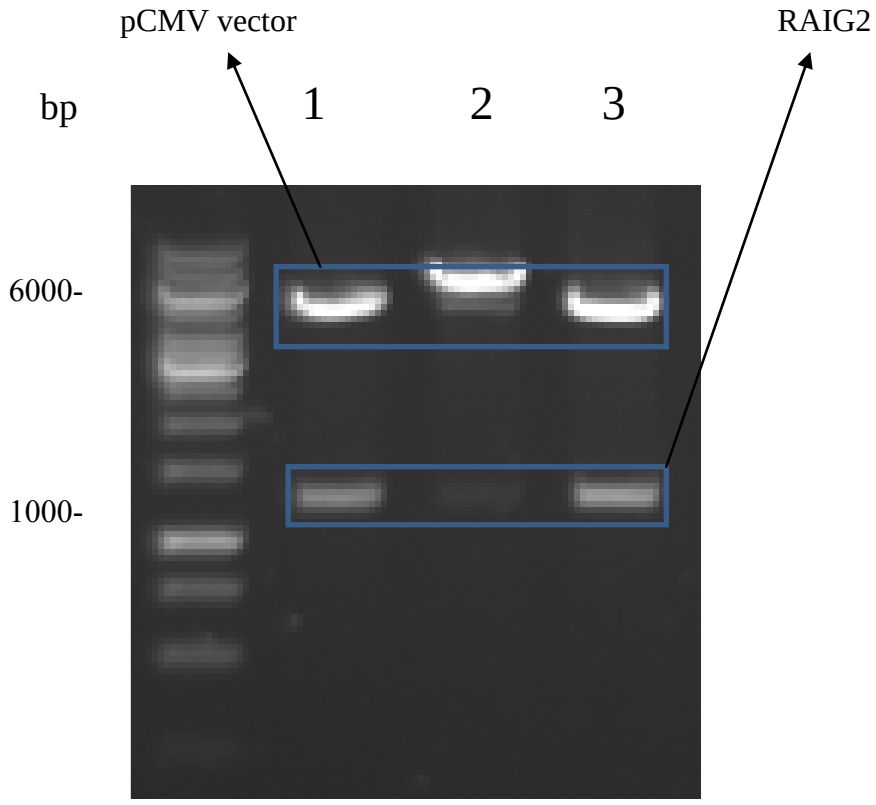


Figure 4.2: Ligation of RAIG2 into the pCMV Vector.

RAIG2/pCMV digested with Sgf1 and Xho1 (Lanes 1-3). 200ng of DNA in each well. Bands observed above 1000bp (RAIG2 1212bp), which indicate the RAIG2 sequence. Bands observed below 5000bp (pCMV vector 4900bp), which indicates the linearized pCMV vector.

4.2 Protein Expression in HEK293 Cells

For the purpose of protein expression, HEK293 Cells were used to express RAIG2, RAIG3 and TPRC4. Following the failure of the cell free expression system to express the RAIGs, mammalian expression using HEK293 cells was undertaken. The sequences of RAIG2, RAIG3 and TPRC4 were all in vectors for mammalian expression, which all promised high quantities of expressed protein.

4.2.1 RAIG2 Expression in HEK293 Cells

To detect RAIG2 expression in HEK293 cells, the FLAG antibody (1:1000 dilution) was used to blot for the presence of full length RAIG2. Different volumes of cells were run on the gel and also untransfected HEK cells were run as a control. The blot contained a strong band just above the protein marker of 36kDa. This confirmed RAIG2 expression as the molecular weight of RAIG2 is 42kDa. Untransfected and transfected HEK cells in PBS were boiled in sample buffer (60 ^{0}C) prior to loading in the gel.

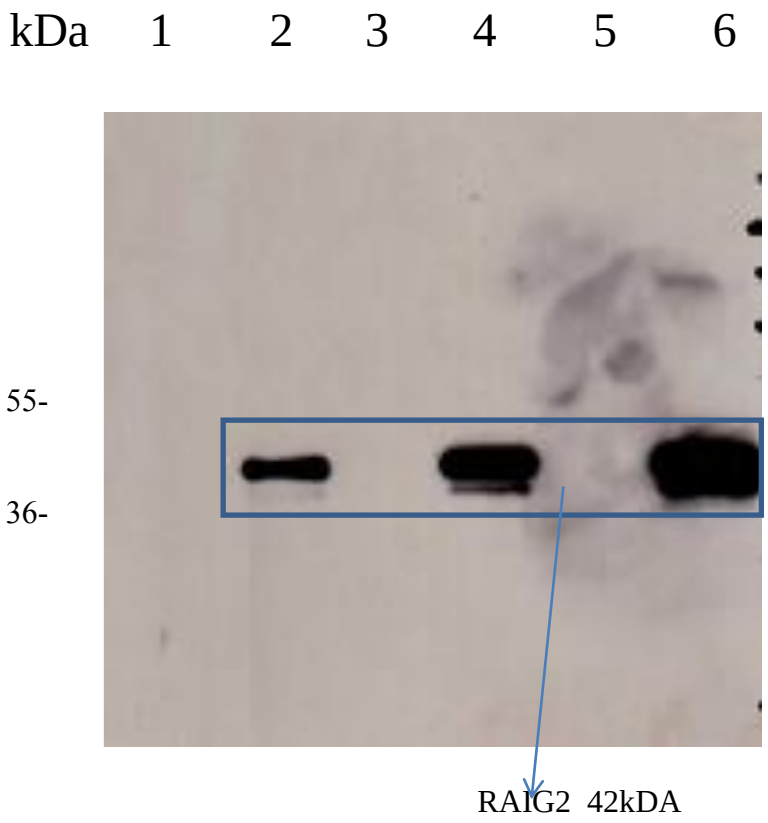


Figure 4.3: Expression of RAIG2 in HEK Cells.

RAIG2 was detected with the FLAG antibody (1:1000). Untransfected HEK cells in PBS (Lane 1), 10µl transfected HEK cells in PBS (Lane 2), 20µl transfected HEK cells in PBS (Lane 4), 30µL transfected HEK cells in PBS (Lane 6). All other lanes are empty. Strong Bands were observed above the 36kda molecular marker (RAIG2 42kda).

4.2.2 RAIG3 Expression in HEK293 Cells

To detect RAIG3 expression in HEK cells, the FLAG antibody (1:1000 dilution) was used to blot for the presence of full length RAIG3. Solubilized transfected HEK293 cells in PBS were applied to the gel and untransfected HEK293 cells in PBS served as the control. The Western blot contained a strong band just below the protein marker of 55kDa. This confirmed RAIG3 expression as the molecular weight of RAIG3 is 52kDa. Untransfected and

transfected HEK293 cells in PBS were boiled in sample buffer (60 ^{0}C) prior to loading in the gel.

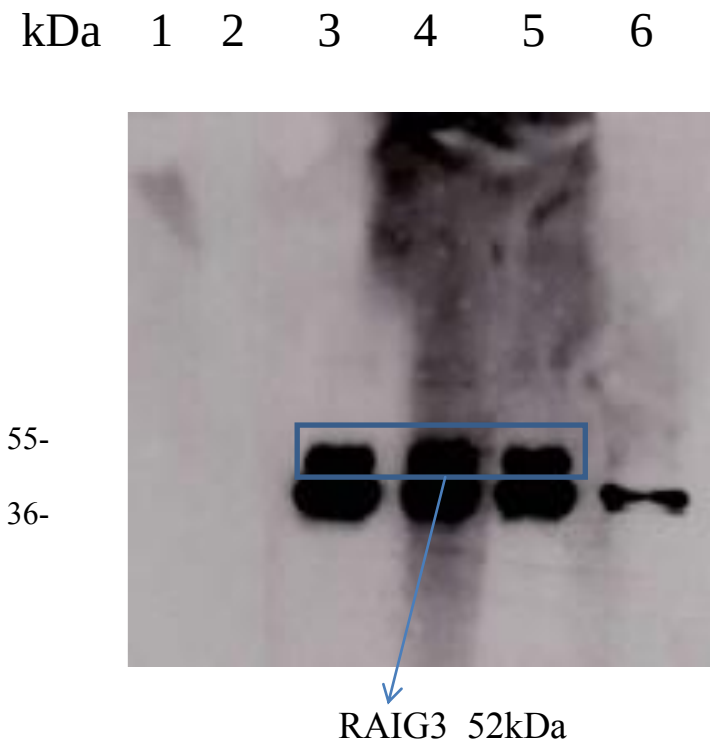


Figure 4.4: Expression of RAIG3 in HEK293 Cells.

Protein was detected with the FLAG antibody (1:1000). Untransfected HEK cells (Lane 1 and 2), 30µl Transfected HEK cells in PBS (boiled in SDS sample buffer) (Lanes 3,4,5,6). Strong Bands were observed below the 55kda molecular marker (RAIG3 52kda).

4.2.3 Analysis of RAIG3-Containing Membranes

Membranes from HEK293 cells, transfected with the RAIG3/pCMV-Entry vector, were blotted with the FLAG antibody (1:1000) to confirm that RAIG3 was expressed in the membrane. As a positive control 30µl of HEK cells in PBS, which previously expressed RAIG3 (see figure 10), was run in lane 1. The FLAG antibody detected many non-specific proteins in the membrane preparation but a strong band did appear below the 55kDa

molecular marker indicating successful RAIG3 membrane localisation (RAIG3 52kDa). Approximately 5µg of the membrane prep was run in lane 2.

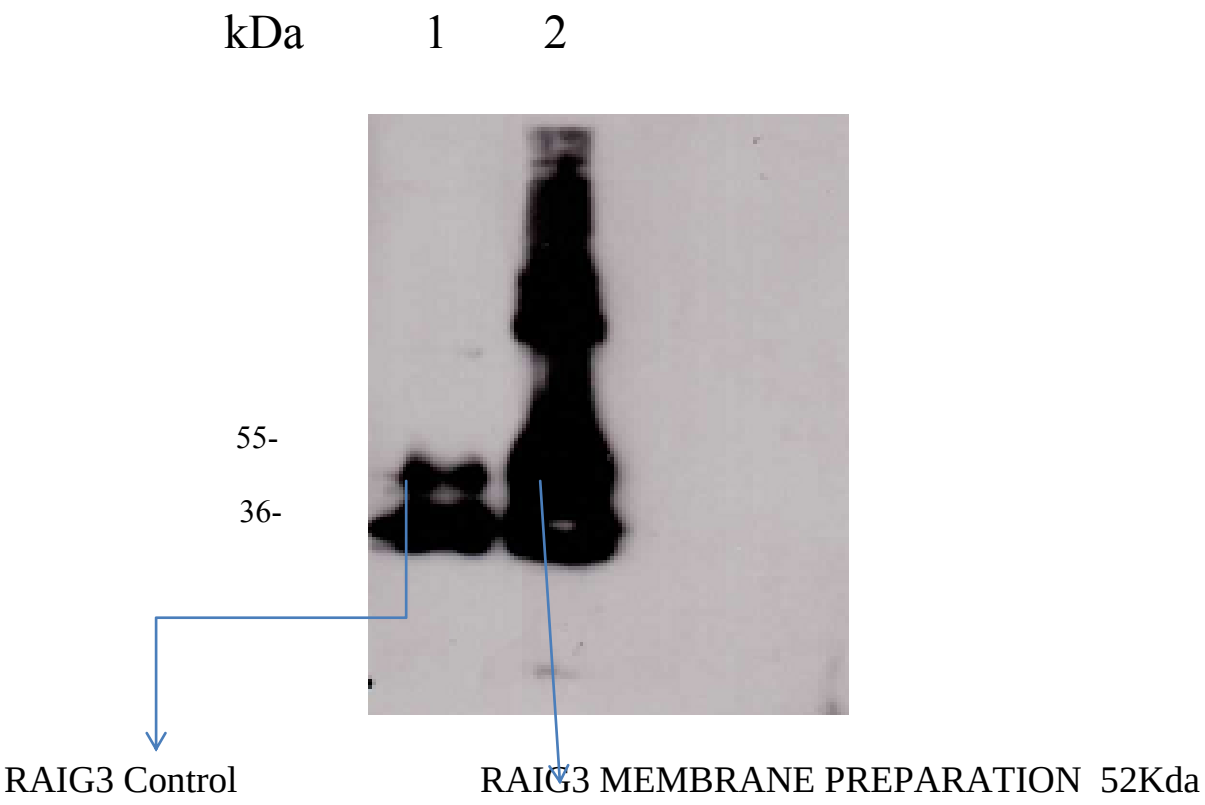


Figure 4.5: Expression of RAIG3 in Isolated HEK293 Membrane Fraction.

FLAG antibody (1:1000) used to detect RAIG3. RAIG3 positive control from previous transfection in HEK cells (Lane 1). 5µg of soluble membranes from membrane prep (Lane 2). Strong band similar to the control appears just below 55KDa in lane 2 (RAIG3 52Kda)

4.2.4 TRPC4 expression in HEK293 cells

To examine TRPC4 Zeta expression in HEK cells, the HA antibody (1:1000 dilution) was used in a Western Blotting experiment to detect the presence of "tagged"-TRPC5. The blot revealed a strong band just below the protein marker of 95kDa in lanes 1 and 3. This confirmed TPRC4 expression as the molecular weight of TPRC4 is 92kDa.

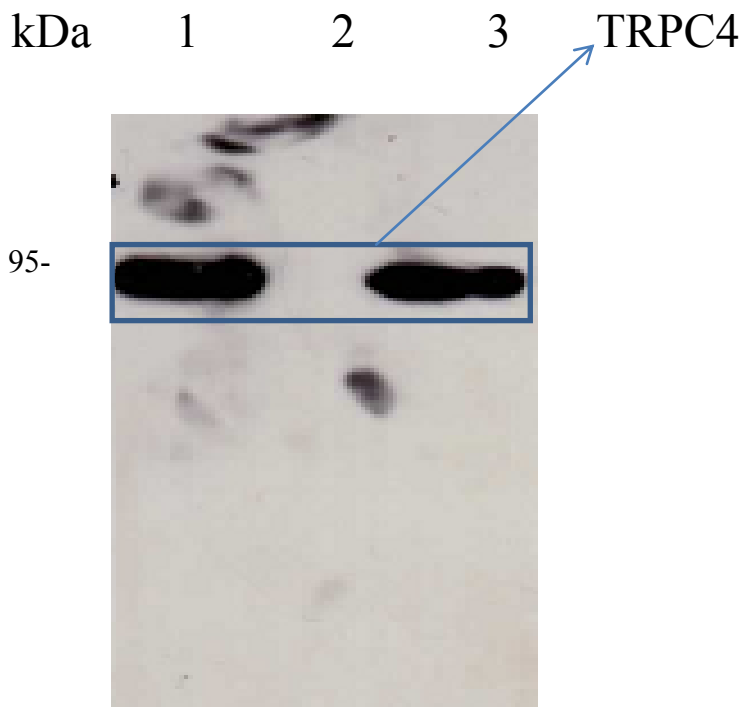


Figure 4.6: Expression of TRPC4 Zeta in HEK Cells.

The HA antibody (1:1000) was used to detect expression of TRPC4. Lane 1 40µl of transfected HEK cells in PBS, boiled in SDS sample buffer. Lane 3 30µl of transfected HEK cells in PBS, boiled in SDS sample buffer. There was no untransfected control used.

4.3 CRBP expression in *E. coli*

The expression of CRBP in bacteria was confirmed by staining the SDS-PAGE gel with page blue. Large quantities of pure CRBP were detected in the 2 elution fractions in lane 5 and 6. The CRBP-GST fusion protein has a molecular weight of 42kDa. Strong bands were visualised with Coomassie Blue above the molecular weight marker of 36kDa. The eluted fractions contained 1.7mg/ml of pure CRBP-GST fusion protein.

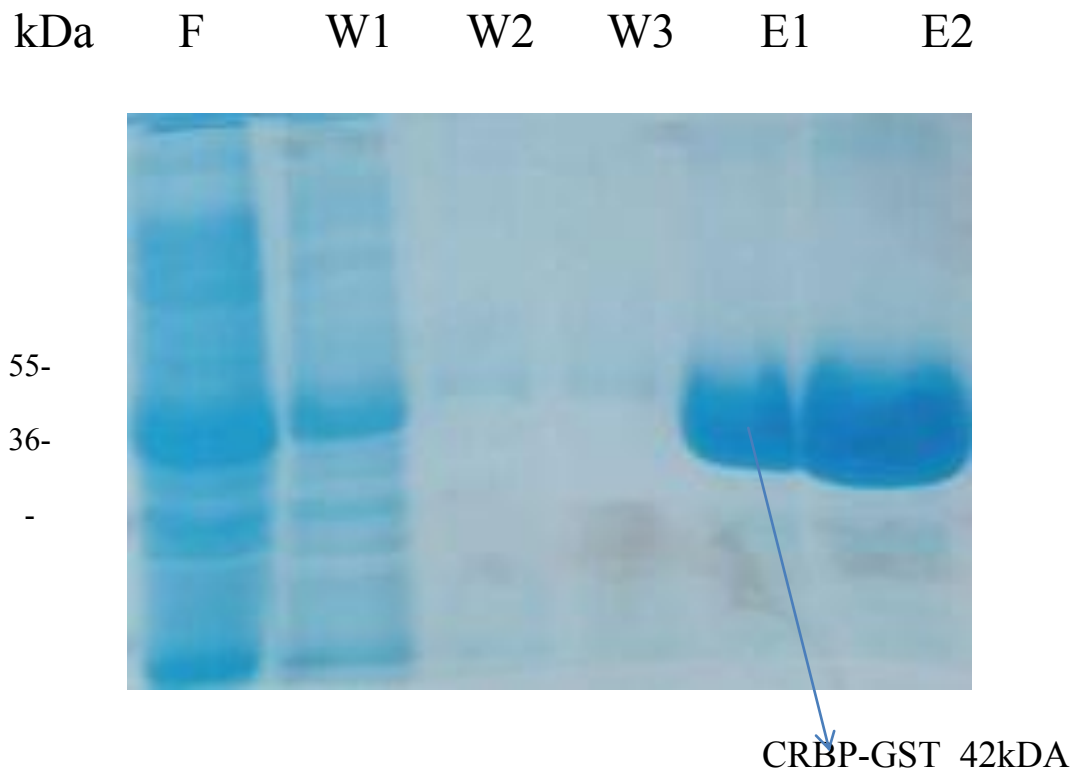


Figure 4.7: Expression and Purification of CRBP.

SDS-PAGE of the flow-through(F), wash (W) and eluted(E) fractions of a soluble E.coli extract applied to Glutathione-Sepharose. Eluted fraction (E) show strong bands above the 35Kda protein marker (CRBP-GST 42kda). CRBP expression and purificartion was a success.

Chapter 5

Protein: protein Interaction Studies

5.1 Introduction

This section describes the series of pull down assays using a novel oil-based assay to determine if the proteins were indeed interacting. Protein: protein interactions were probed using a pull-down assay adapted to accommodate the possible low affinity of RBP for RAIG2 or RAIG3 and CRBP for TPRC4. A traditional wash step would potentially disrupt the receptor: ligand complex, and was therefore eliminated and substituted with centrifugation through a non-aqueous oil layer to minimize loss of any observable receptor: ligand complex through dissociation.

5.1.1 Oil Pull-downs with RAIG2

This aspect of the study involved a pull down assay using a c-Myc antibody immobilised on an agarose resin. The RAIG2 protein contained a C-Myc tag. An RBP antibody (1:1000 dilution) was used to detect any RBP which co-sediments with the RAIG2-bound resin. C-Myc resin (50µl) was run on the first lane of the gel as the RBP antibody appeared non-specifically cross-reacting with the c-Myc antibody. The third lane contained the c-Myc resin (with RAIG2 attached) pulled down through the oil. There was no band at the appropriate size for RBP (21kDa). In this lane similar bands appeared as in the first lane containing just the c-Myc resin. The fifth lane contained the aqueous layer of the oil pull down, containing proteins that did not bind to the c-Myc resin which contained RAIG2. This lane showed a significant band at 21kDa corresponding to RBP. This shows that RBP did not bind to the c-Myc resin or RAIG2. A negative control to see if RBP bound to the c-Myc resin was also run. Lane 7 shows that RBP did not pull down through the oil with the c-Myc resin in the negative control, with RBP only detected in the aqueous layer in lane 9.

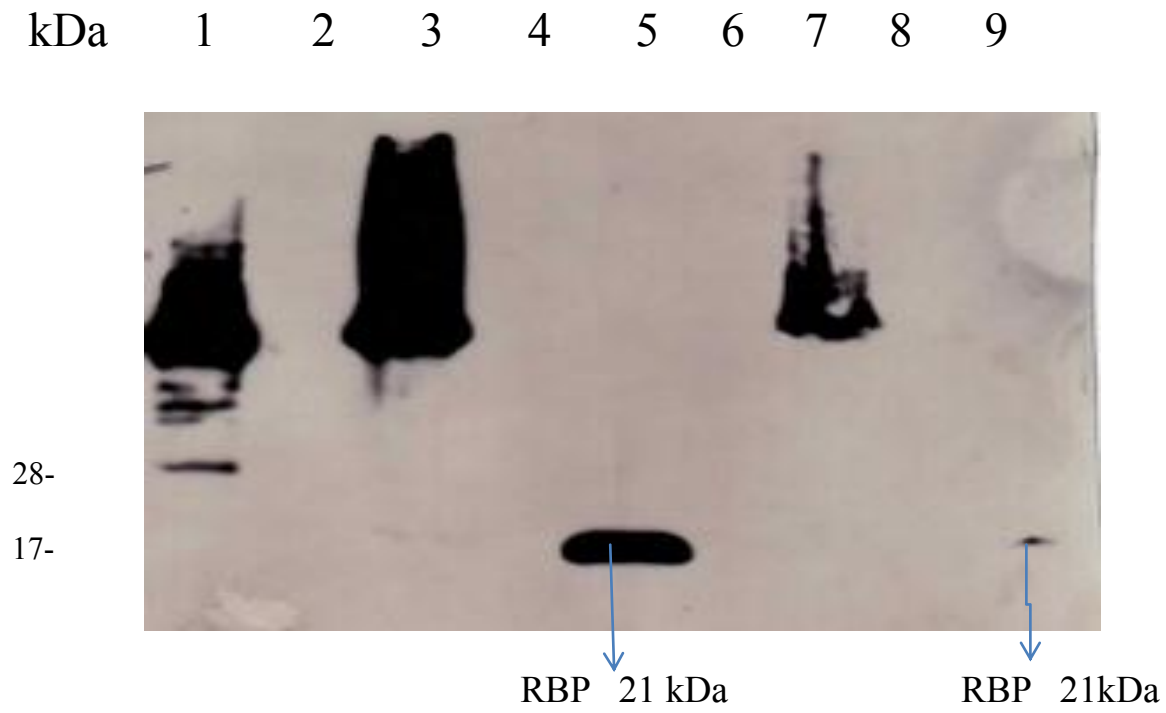


Figure 5.1: Analysis of RBP in Pull-downs with RAIG2.

RBP antibody (1:1000) was used to detect RBP in a pull-down interaction study. C-Myc resin (Lane 1), c-Myc resin, incubated with RAIG2 pulled down through oil (Lane 3), aqueous layer after oil pull down (Lane 5), c-Myc resin alone pulled down through oil of negative control (Lane 7), aqueous layer after oil pull down of negative control, all other lanes are blank. Blot shows that RBP (21kda) was only detected in the aqueous layer of pull down and did not pull-down with RAIG2.

5.1.2 Analysis of RAIG2 in Pull-downs with RBP

In order to confirm the presence of RAIG2, the same membrane as above was stripped and reprobed with the FLAG antibody (1:1000 dilution). As expected a band for RAIG2 was detected at 42kDa only in in lane 3. This lane contained RAIG2 which was pulled down through the oil by the c-Myc resin. These two blots prove that there is no interaction between RAIG2 and RBP. The c-Myc antibody pulled down RAIG2 with no RBP attached.

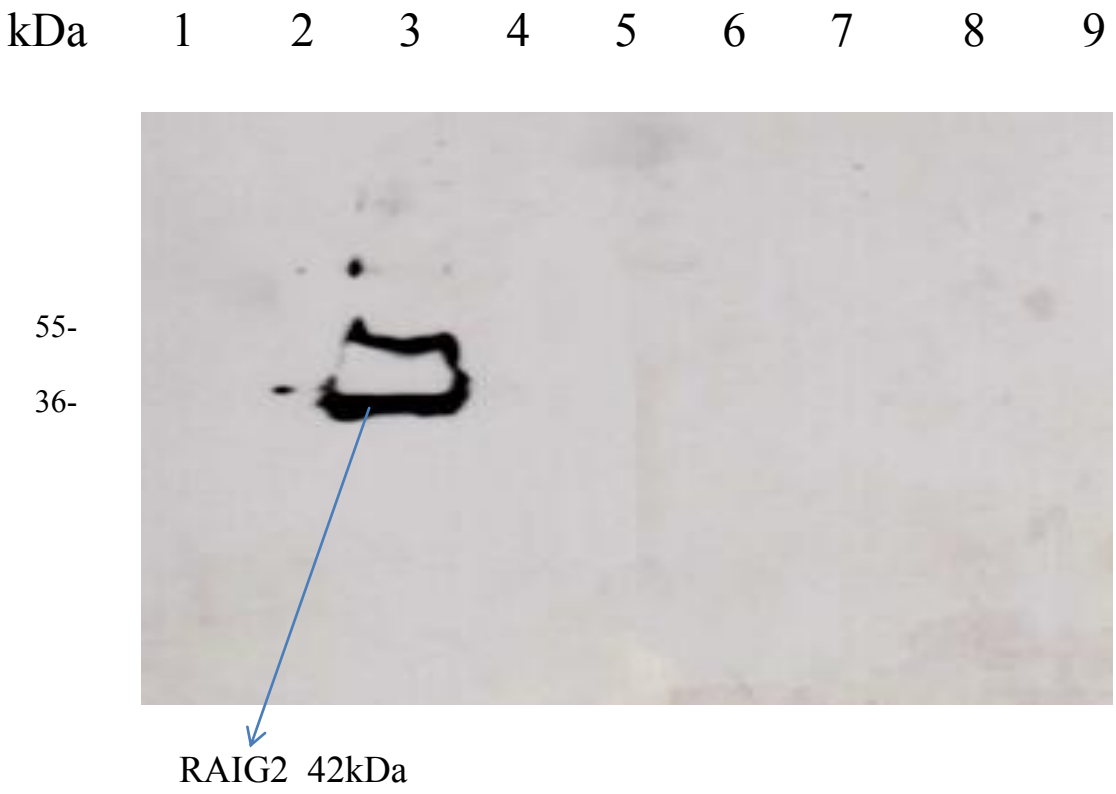


Figure 5.2: Analysis of RAIG2 in Pull-downs with RBP.

Western Blot for RAIG2 using FLAG antibody (1:1000). C-Myc resin (Lane 1), c-Myc-resin in the presence of RAIG2 pulled down through oil (Lane 3), aqueous layer after oil pull down (Lane 5), resin alone pulled down through oil (Lane 7). All lanes except 3 which contains the RAIG2, are blank.

5.1.3 Oil Pull-downs with RAIG3

This aspect of the study involved a pull down assay using a c-Myc antibody immobilised on an agarose resin. The RAIG3 protein contained a c-Myc tag. An RBP antibody (1:1000 dilution) was used to detect any RBP which co-sediments with the RAIG3-bound resin. The antibody detected a band at 21kDa (RBP) in Lanes 2 and 8. Lane 2 contained 100ng of RBP as a positive control. Lane 5 contained the c-Myc-resin-RAIG3 complex which was pulled down through the oil in the assay. Lane 8 contained the aqueous layer of the pull down

containing the proteins which did not bind to the RAIG3-c-Myc-resin complex. A strong band for RBP was detected in the aqueous layer. These results prove that no RBP is binding to RAIG3 or the c-Myc resin. A negative control to see if RBP was binding to the c-Myc resin, in the absence of RAIG3, was not necessary. The RBP antibody did not bind to the c-Myc resin as it did with the RBP-RAIG2 pull-down.

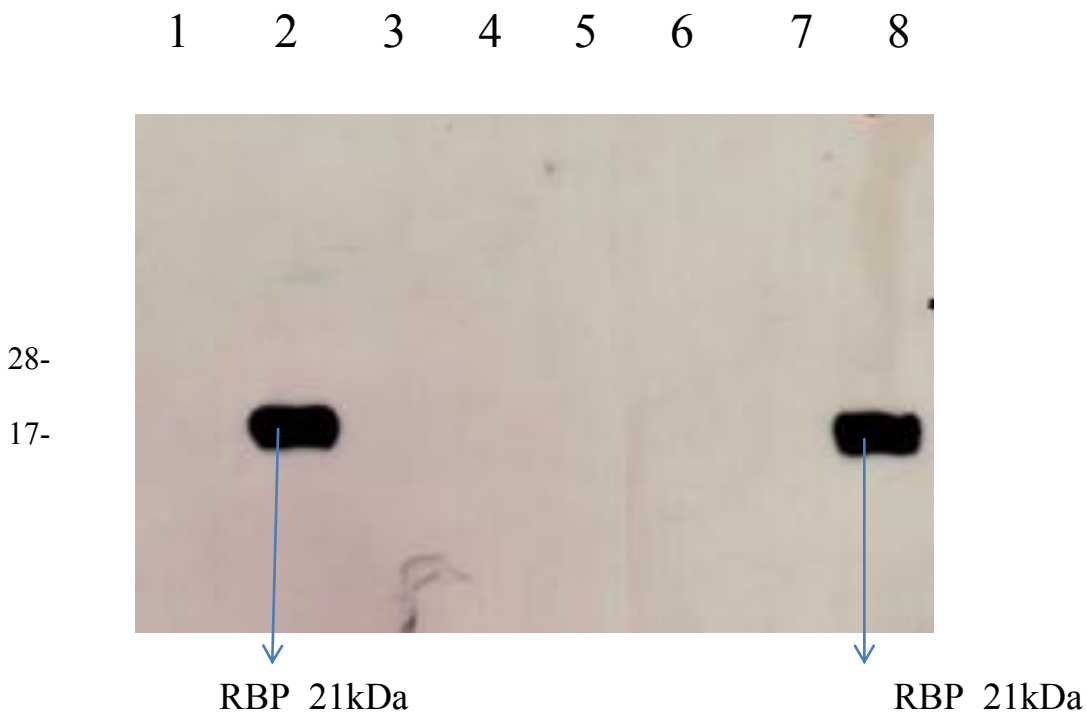


Figure 5.3: Analysis of RBP in Pull-downs with RAIG3.

Western Blot (1:1000) to detect RBP in a RAIG3 Pull-down assay . RAIG3 control (Lane 1), RBP control (Lane 2), c-Myc resin pulled down through oil (Lane 5), aqueous layer after oil pull down (Lane 8), all other lanes are blank. Blot shows that RBP (21kda) was only detected in the aqueous layer of pull down. This means that no RBP is binding to the RAIG3 in the resin which should be in lane 5.

5.1.4. Analysis of RAIG3 in Pull-downs with RBP

In order to confirm the presence of RAIG3 in the pull-down experiment, the same membrane as above was stripped and reprobed with the FLAG antibody (1:1000 diluton). As expected a band for RAIG3 was detected at 52kDa in lane 5. This lane contained RAIG3 which was pulled down through the oil by the c-Myc resin. These two blots prove that there is no interaction between RAIG3 and RBP. The c-Myc resin pulled down RAIG3 with no RBP attached.

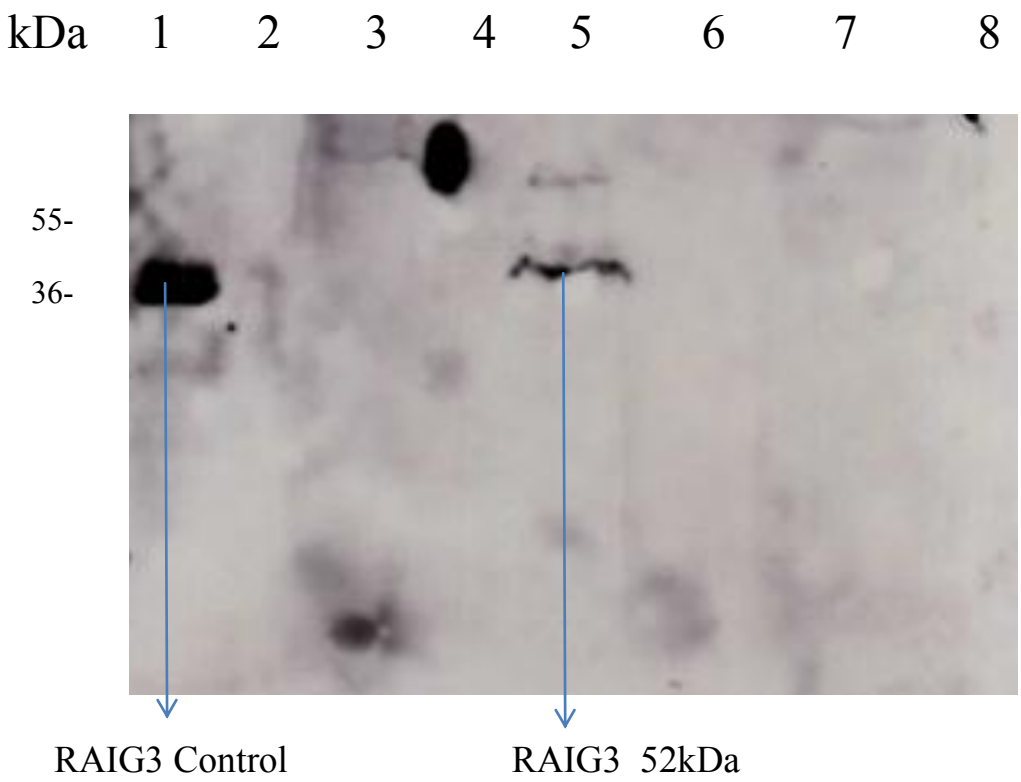


Figure 5.4: Analysis of RAIG3 in Pull-downs with RBP.

Western Blot for the presence of RAIG3. RAIG3 control (Lane 1), RBP control (Lane 2), c-Myc resin pulled down through oil (Lane 5), aqueous layer after oil pull down (Lane 8), all other lanes are blank. RAIG3 (52kda) in lane 5 was pulled down through the oil with the c-Myc resin.

5.1.5 CRBP Binding Capacity of TRPC4 Zeta

The HA antibody (1:1000 dilution) was used to detect TRPC4 Zeta in a standard pull down interaction assay between CRBP and TRPC4. The antibody detected a band at 92kDa (TRPC4) in only the first lane of the blot. This lane contained the supernatant (S) after the first centrifugation step, before the resin was washed. This means that TRPC4 did not bind to the GST resin containing CRBP. The other lanes contain wash and elution steps from the pull down and the blot shows that there is no TRPC4 in these lanes. This shows that there is not a strong interaction between CRBP and TRPC4.

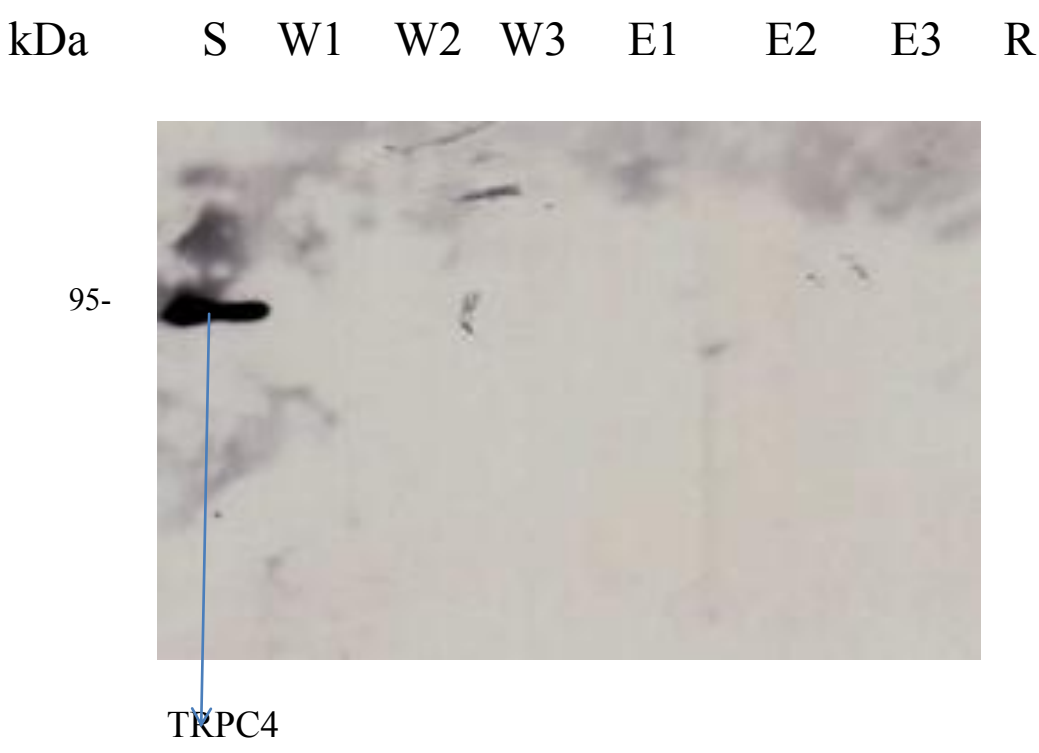


Figure 5.5: Analysis of TRPC4 in Pull-downs with CRBP

Blot for TRPC4 Zeta using the HA antibody (1:1000). TRPC4 protein band observed in the supernatant (S) which shows that the TRPC4 protein did not bind to the GST resin containing the CRBP protein in any of the eluted fractions (E). The TRPC4 protein was in the supernatant after the first centrifugation step with no TRPC4 in any of the washes and elutions.

The standard pull-down was done to see if there was a possible strong interaction between TRPC4 and CRBP. The blots show that TRPC4 did not cum through in the elution step and was in the supernatant (S) after the first centrifugation step before the resin was washed 3 times with PBS.

5.1.6 Analysis of CRBP in Pull-downs with TRPC4

In order to visualise the CRBP protein, the same membrane as above was stripped and reprobed with the CRBP antibody (1:500 diluton). Bands were detected just over the 36kDa molecular marker in all the lanes on the blot. The CRBP-GST fusion protein has a molecular weight of 42kDa. The blot also proved that CRBP-GST bound to the glutathione resin and was eluted off in each elution step (E). During each wash step (W), CRBP was detected but there was still sufficient CRBP in the elution steps to bind TRPC4. This proves that there is no strong interaction between CRBP and TRPC4.

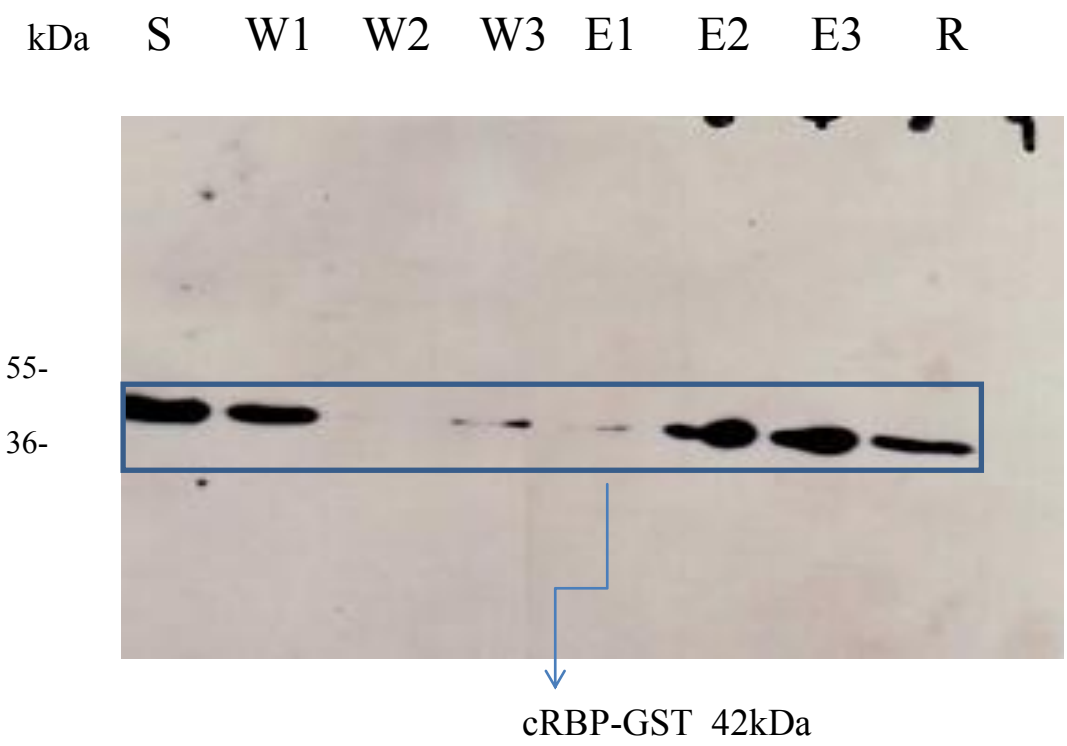


Figure 5.6: Analysis of CRBP in Pull-downs with TRPC4.

Blot for CRBP using the CRBP antibody (1:500). Bands observed above 35kda (CRBP 42kda). Combining the previous blot for TRPC4, it shows that TRPC4 Zeta is not binding to CRBP. CRBP is in the eluted fractions, with TRPC4 only seen in the first centrifugation step before the resin was washed with PBS.

5.1.7 Oil pull down involving TRPC4 and CRBP

To see if there was a weak interaction between TRPC4 and CRBP, the oil-based pull-down was used to determine if the two proteins were interacting. This aspect of the study involved a pull down assay using a HA antibody immobilised on an agarose resin. The TRPC4 protein contained a HA tag. A CRBP antibody (1:500 dilution) was used to detect any CRBP which co-sediments with the TRPC4-bound resin. The CRBP-GST fusion protein has a molecular weight of 42kDa. Bands were detected just over the 36kDa in lane 5 and lane 11. Lane 5 indicates the aqueous layer of the pull down. This is the layer that contains material that did not bind to the TRPC4 protein and HA resin, which was run in lane 2. A negative control to see if CRBP bound to the HA resin was also run. Lane 8 shows that CRBP did not pull down through the oil with the HA resin in the negative control. Lane 11 shows the aqueous layer of the negative control, with a band for CRBP.

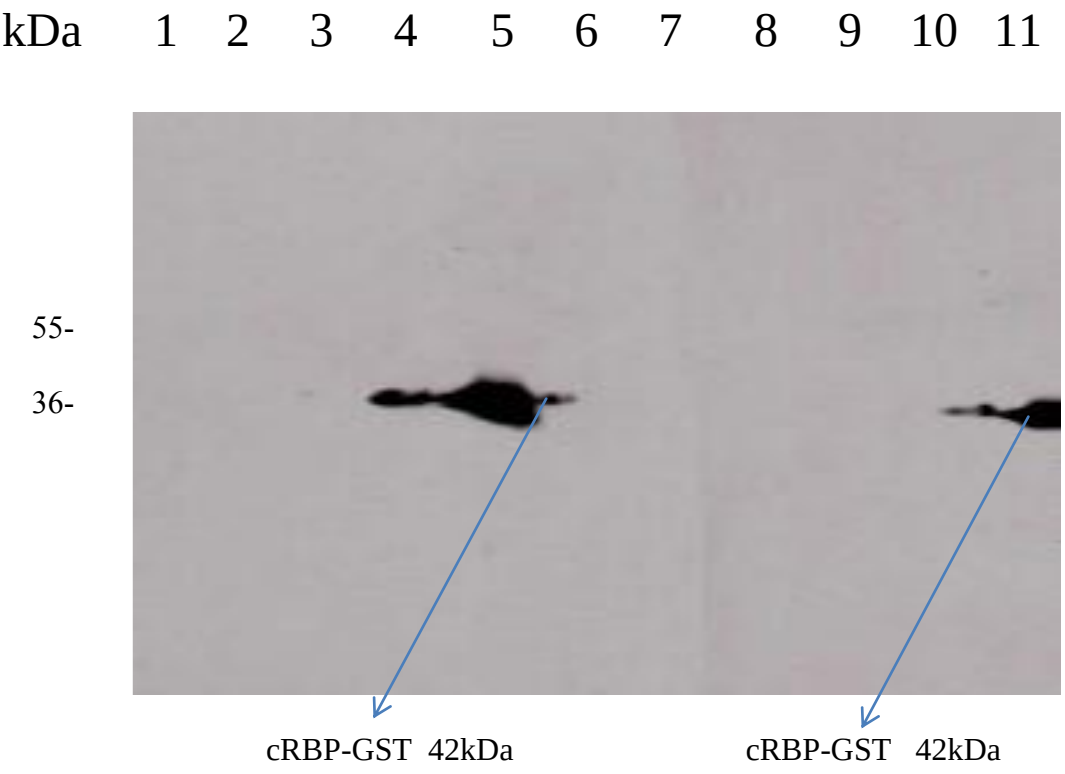


Figure 5.7: Analysis of CRBP in Oil Pull-downs with TRPC4.

Western Blot of Fractions from an Oil Pull-down Assay. Membrane was probed with the CRBP antibody to detect CRBP. CRBP-GST (42kDa) was detected in lanes 5 and 11. Both these lanes contain the aqueous layer of the pull down assay. HA resin, incubated with TRPC4 pulled down through oil (Lane 2), aqueous layer after oil pull down (Lane 5), negative control containing c-Myc resin alone pulled down through oil (Lane 8), aqueous layer after oil pull down of negative control (Lane 11), all other lanes are blank.

5.1.8 Analysis of TRPC4 in Pull-downs with CRBP

In order to visualise the TRPC4 band, the membrane was stripped and reprobed with the HA antibody (1:1000 diluton). The second lane contained the HA resin (with TRPC4 attached) pulled down through the oil, which shows a band below 95kDa (TRPC4 92Kda). The fifth lane contained the aqueous layer of the oil pull down, containing proteins that did not bind to the HA resin. There is a slight band in lane 5 below 95kDa indicative of excess TRP. The negative control (HA resin and CRBP) does not pick up a band for TRP as expected. The HA antibody appeared non-specifically cross-reacting with the HA resin similar to the RBP antibody and the c-Myc resin (see figure 5.1). This produces two additional bands in lane 2 and 8. These two blots together show there is no weak interaction between CRBP and TRPC4. The HA resin pulled down TRPC4 with no CRBP attached.

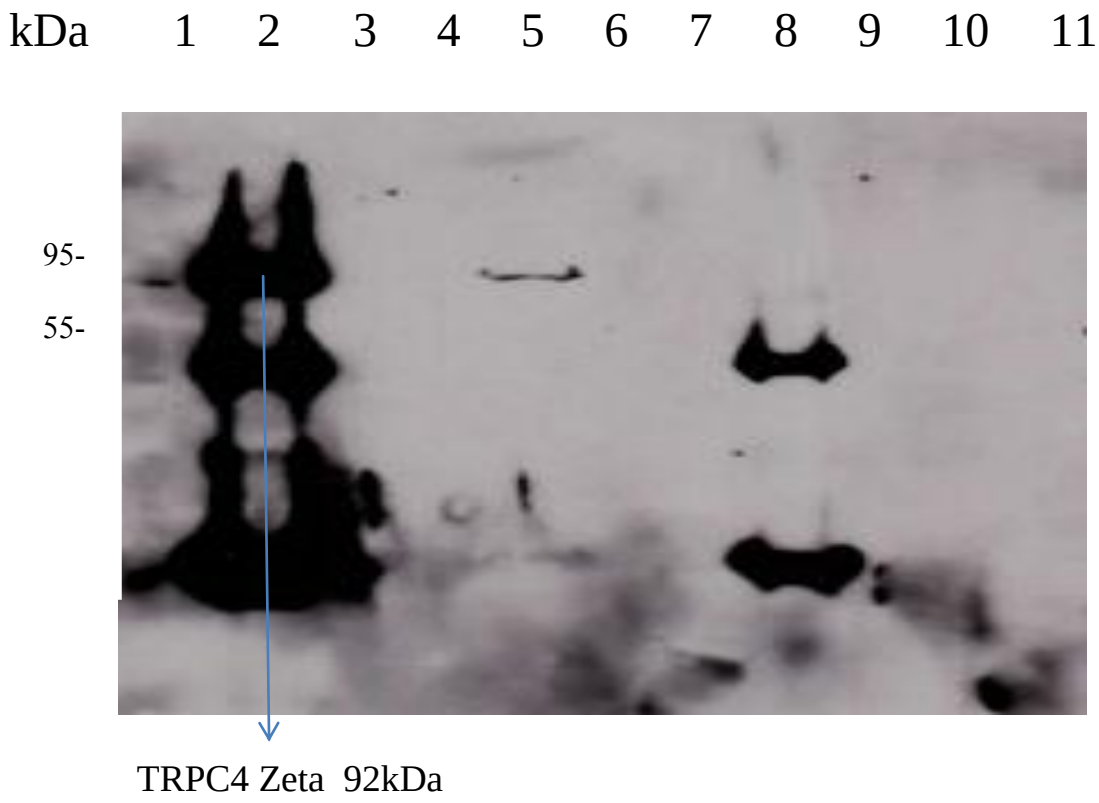


Figure 5.8: Analysis of TRPC4 in Pull-downs with CRBP.

Western Blot of a Pull-down assay using TRPC4 immobilised on a HA Resin. Membrane blotted with HA antibody to detect TRPC4 Zeta. Western Blot of Fractions from an Oil Pull-down Assay. HA resin, incubated with TRPC4 pulled down through oil (Lane 2), aqueous layer after oil pull down (Lane 5), negative control containing c-Myc resin alone pulled down through oil (Lane 8), aqueous layer after oil pull down of negative control (Lane 11), all other lanes are blank.

Chapter 6
Discussion

DISCUSSION

The aim of this project was to follow up on possible hits from yeast two hybrid work to determine if there were protein interactions between (i) RBP and RAIG2 (ii) RBP and RAIG3 and (iii) CRBP and TRPC4 Zeta ion channel. Interaction between these proteins could have a serious role in insulin resistance and type 2 diabetes.

The original strategy focused on expressing RAIG2 and RAIG3 in the MembraneMax^{TM} cell -free system. Molecular biology work centred on creating expression vectors suitable for this system. This involved PCR, restriction digests and subcloning. Expression of RAIG2, RAIG3 and TPRC4 was also attempted in HEK293 cells. This involved more molecular work to get the RAIG2 sequence subcloned into a mammalian expression vector. The RAIG3 and TRPC4 sequences were already in mammalian expression vectors so no molecular work was required. Also, bacterial expression and purification of CRBP-GST fusion protein was undertaken with 1.7mg/ml of pure protein produced. The final section focused on performing a series of pull down assays using a novel oil-based assay to determine if the proteins were indeed interacting.

Even though the results were negative and the proteins didn't interact, these results have eradicated these bait proteins from being involved in insulin resistance and type 2 diabetes. These results have primarily put the focus back on RBP and STRA6 in elucidating the mechanism for this disease. The membrane yeast two hybrid system is known to throw up

many false positives which often leads to negative results when determining molecular interactions.

The TRP channel is a highly studied protein and a lot is already known about its function and role in the cell. As for the RAIG protein, little is still known about this novel GPCR. Discovery of a ligand or a particular associated G-protein will help in elucidating the function of this GPCR. These results have helped further deorphanise the RAIG receptor and eliminate RBP as a possible ligand. It expressed in high amounts and solubilized quite well which can often be a problem with GPCRs. The TRPC4 protein also expressed very well and solubilized in appropriate buffer.

Cell free expression of the RAIGs did not occur but the system will be optimized for future work. The reason why the expression did not occur is still very puzzling. The vectors for each protein contained all the necessary machinery for gene expression in this system. There has been mixed results in the past for this system with some constructs expressing well and others not expressing at all. Cell-free expression of membrane proteins is not however, without its difficulties and it is thought that in both methods, requirement of insertion into the lipid bilayer to ensure correct folding and function may be the stumbling point (Cappuccio *et al.*, 2008).

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Appendix 1 Nucleotide and Amino Acid Sequences

1.1 Full-Length Human RAIG2 UniProt Entry Q9NZHO (GPC5B_HUMAN)

Nucleotide Sequence

ATGTTCGTGGCATCAGAGAGAAAGATGAGAGCTCACCAGGTGCTCACCTTCCTCCTGCTCTTCGTGATCACCTCG
GTGGCCTCTGAAAACGCCAGCACATCCCGAGGCTGTGGGCTGGACCTCCTCCCTCAGTACGTGTCCCTGTGCGAC
CTGGACGCCATCTGGGGCATTGTGGTGGAGGCGGTGGCCGGGGCGGGCGCCCTGATCACACTGCTCCTGATGCTC
ATCCTCCTGGTGCGGCTGCCCTTCATCAAGGAGAAGGAGAAGAAGAGCCCTGTGGGCCTCCACTTTCTGTTCCTC
CTGGGGACCCTGGGCCTCTTTGGGCTGACGTTTGCCTTCATCATCCAGGAGGACGAGACCATCTGCTCTGTCCGC
CGCTTCCTCTGGGGCGTCCTCTTTGCGCTCTGCTTCTCCTGCCTGCTGAGCCAGGCATGGCGCGTGCGGAGGCTG
GTGCGGCATGGCACGGGCCCCGCGGGCTGGCAGCTGGTGGGCCTGGCGCTGTGCCTGATGCTGGTGCAAGTCATC
ATCGCTGTGGAGTGGCTGGTGCTCACCGTGCTGCGTGACACAAGGCCAGCCTGCGCCTACGAGCCCATGGACTTT
GTGATGGCCCTCATCTACGACATGGTACTGCTTGTGGTCACCCTGGGGCTGGCCCTCTTCACTCTGTGCGGCAAG
TTCAAGAGGTGGAAGCTGAACGGGGCCTTCCTCCTCATCACAGCCTTCCTCTCTGTGCTCATCTGGGTGGCCTGG
ATGACCATGTACCTCTTCGGCAATGTCAAGCTGCAGCAGGGGGATGCCTGGAACGACCCCACCTTGGCCATCACG
CTGGCGGCCAGCGGCTGGGTCTTCGTCATCTTCCACGCCATCCCTGAGATCCACTGCACCCTTCTGCCAGCCCTG
CAGGAGAACACGCCCAACTACTTCGACACGTCGCAGCCCAGGATGCGGGAGACGGCCTTCGAGGAGGACGTGCAG
CTGCCGCGGGCCTATATGGAGAACAAGGCCTTCTCCATGGATGAACACAATGCAGCTCTCCGAACAGCAGGATTT
CCCAACGGCAGCTTGGGAAAAAGACCCAGTGGCAGCTTGGGGAAAAGACCCAGCGCTCCGTTTAGAAGCAACGTG
TATCAGCCAACTGAGATGGCCGTCGTGCTCAACGGTGGGACCATCCCAACTGCTCCGCCAAGTCACACAGGAAGA
CACCTTTGG

-Stop codon not included.

Amino Acid Sequence

MFVASERKMRAHQVLTFLLLFVITSVASENASTSRGCGLDLLPQYVSLCDLDAIWGIVVEAVAGAGALITLLLML
ILLVRLPFIKEKEKKSPVGLHFLFLLGTLGLFGLTFAFIIQEDETICSVRRFLWGVLFALCFSCLLSQAWRVRRL
VRHGTGPAGWQLVGLALCLMLVQVIIAVEWLVLTVLRDTRPACAYEPMDFVMALIYDMVLLVVTLGLALFTLCGK
FKRWKLNGAFLLITAFLSVLIWVAWMTMYLFGNVKLQQGDAWNDPTLAITLAASGWVFVIFHAIPEIHCTLLPAL
QENTPNYFDTSQPRMRETAFEEDVQLPRAYMENKAFSMDEHNAALRTAGFPNGSLGKRPSGSLGKRPSAPFRSNV
YQPTEMAVVLNGGTIPTAPPSHTGRHLW

1.2 Full-Length Human RAIG3 UniProt Entry Q9NQ84 (GPC5C_HUMAN)

Nucleotide Sequence

ATGCGGGGGCGTGGCAGTCAACAGCAACAACCCACACGCCGGCAGGGCCAGAAACTCCCATCTCCCTCACCAGCC
GGAAAGTACGAGTCGGCTCAGCCTGGAGGGACCCAACCAGAGCCTGGCCTGGGAGCCAGGATGGCCATCCACAAA
GCCTTGGTGATGTGCCTGGGACTGCCTCTCTTCCTGTTCCCAGGGGCCTGGGCCCAGGGCCATGTCCCACCCGGC
TGCAGCCAAGGCCTCAACCCCCTGTACTACAACCTGTGTGACCGCTCTGGGGCGTGGGGCATCGTCCTGGAGGCC
GTGGCTGGGGCGGGCATTGTCACCACGTTTGTGCTCACCATCATCCTGGTGGCCAGCCTCCCCTTTGTGCAGGAC
ACCAAGAAACGGAGCCTGCTGGGGACCCAGGTATTCTTCCTTCTGGGGACCCTGGGCCTCTTCTGCCTCGTGTTT
GCCTGTGTGGTGAAGCCCGACTTCTCCACCTGTGCCTCTCGGCGCTTCCTCTTTGGGGTTCTGTTCGCCATCTGC
TTCTCTTGTCTGGCGGCTCACGTCTTTGCCCTCAACTTCCTGGCCCGGAAGAACCACGGGCCCCGGGGCTGGGTG
ATCTTCACTGTGGCTCTGCTGCTGACCCTGGTAGAGGTCATCATCAATACAGAGTGGCTGATCATCACCCTGGTT
CGGGGCAGTGGCGAGGGCGGCCCTCAGGGCAACAGCAGCGCAGGCTGGGCCGTGGCCTCCCCCTGTGCCATCGCC
AACATGGACTTTGTCATGGCACTCATCTACGTCATGCTGCTGCTGCTGGGTGCCTTCCTGGGGGCCTGGCCCGCC
CTGTGTGGCCGCTACAAGCGCTGGCGTAAGCATGGGGTCTTTGTGCTCCTCACCACAGCCACCTCCGTTGCCATA

TGGGTGGTGTGGATCGTCATGTATACTTACGGCAACAAGCAGCACAACAGTCCCACCTGGGATGACCCCACGCTG
GCCATCGCCCTCGCCGCCAATGCCTGGGCCTTCGTCCTCTTCTACGTCATCCCCGAGGTCTCCCAGGTGACCAAG
TCCAGCCCAGAGCAAAGCTACCAGGGGGACATGTACCCCACCCGGGGCGTGGGCTATGAGACCATCCTGAAAGAG
CAGAAGGGTCAGAGCATGTTCGTGGAGAACAAGGCCTTTTCCATGGATGAGCCGGTTGCAGCTAAGAGGCCGGTG
TCACCATACAGCGGGTACAATGGGCAGCTGCTGACCAGTGTGTACCAGCCCACTGAGATGGCCCTGATGCACAAA
GTTCCGTCCGAAGGAGCTTACGACATCATCCTCCCACGGGCCACCGCCAACAGCCAGGTGATGGGCAGTGCCAAC
TCGACCCTGCGGGCTGAAGACATGTACTCGGCCCAGAGCCACCAGGCGGCCACACCGCCGAAAGACGGCAAGAAC
TCTCAGGTCTTTAGAAACCCCTACGTGTGGGAC

-Stop codon not included.

Amino Acid Sequence

MRGRGSQQQQPTRRQGQKLPSPSPAGKYESAQPGGTQPEPGLGARMAIHKALVMCLGLPLFLFPGAWAQGHVPPG
CSQGLNPLYYNLCDRSGAWGIVLEAVAGAGIVTTFVLTIILVASLPFVQDTKKRSLLGTQVFFLLGTLGLFCLVF
ACVVKPDFSTCASRRFLFGVLFAICFSCLAAHVFALNFLARKNHGPRGWVIFTVALLLTLVEVIINTEWLIITLV
RGSGEGGPQGNSSAGWAVASPCAIANMDFVMALIYVMLLLLGAFLGAWPALCGRYKRWRKHGVFVLLTTATSVAI
WVVWIVMYTYGNKQHNSPTWDDPTLAIALAANAWAFVLFYVIPEVSQVTKSSPEQSYQGDMYPTRGVGYETILKE
QKGQSMFVENKAFSMDEPVAAKRPVSPYSGYNGQLLTSVYQPTEMALMHKVPSEGAYDIILPRATANSQVMGSAN
STLRAEDMYSAQSHQAATPPKDGKNSQVFRNPYVWD

1.3 Full-Length Human TRPC4 UniProt Entry Q9UBN4 (TRPC4_HUMAN)

atggctcagttctattacaaaagaaatgttaatgctccctatagagaccgcatccctctaaggatagtaagagca

gaatcagaactctcgccatcagaaaaagcctacttgaatgctgtggaaaagggagattatgccagtgtcaagaaa

tccctagaggaagctgaaatttatttaaaatcaatattaattgcattgatcctctcggaagaactgctctcctc

attgcaattgaaaatgagaacttggagctcatcgaactactcttaagctttaatgtctatgttggagatgctcta

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ggaaagagagtgtgtccattcaagtcagagaaggtggtggtggaggacacggttcctataataccaaaggagaaa

catgcaaaagaagaggactctagtatagactatgatctaaacctcccagacacagtcacccacgaagattacgtg

accacaagattg

-Stop codon not included.

Amino Acid Sequence

MAQFYYKRNVNAPYRDRIPLRIVRAESELSPSEKAYLNAVEKGDYASVKKSLEEAEIYFKININCIDPLGRTALL
IAIENENLELIELLLSFNVYVGDALLHAIRKEVVGAVELLLNHKKPSGEKQFVAQPNCQQLLASRWYDEFPGWRR
RHWAVKMVTCFIIGLLFPVFSVCYLIAPKSPLGLFIRKPFIKFICHTASYLTFLFLLLLASQHIDRSDLNRQGPP
PTIVEWMILPWVLGFIWGEIKQMWDGGLQDYIHDWWNLMDFVMNSLYLATISLKIVAFVKYSALNPRESWDMWHP
TLVAEALFAIANIFSSLRLISLFTANSHLGPLQISLGRMLLDILKFLFIYCLVLLAFANGLNQLYFYYEETKGLT
CKGIRCEKQNNAFSTLFETLQSLFWSIFGLINLYVTNVKAQHEFTEFVGATMFGTYNVISLVVLLNMLIAMMNNS
YQLIADHADIEWKFARTKLWMSYFEEGGTLPTPFNVIPSPKSLWYLIKWIWTHLCKKKMRRKPESFGTIGRRAAD
NLRRHHQYQEVMRNLVKRYVAAMIRDAKTEEGLTEENFKELKQDISSFRFEVLGLLRGSKLSTIQSANASKESSN
SADSDEKSDSEGNSKDKKKNFSLFDLTTLIHPRSAAIASERHNISNGSALVVQEPPREKQRKVNFVTDIKNFGLF
HRRSKQNAAEQNANQIFSVSEEVARQQAAGPLERNIQLESRGLASRGDLSIPGLSEQCVLVDHRERNTDTLGLQV
GKRVCPFKSEKVVVEDTVPIIPKEKHAKEEDSSIDYDLNLPDTVTHEDYVTTRL