FUNCTIONAL HUMAN CANNABINOID RECEPTOR 2 FROM *ESCHERICHIA COLI*

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The Cannabinoid Receptor 2 (CB2), a member of the endocannabinoid system belongs to the Rhodopsin family of G-Protein Coupled Receptors (GPCRs). It is expressed mainly in the immune cells and exerts immunomodulatory roles in normal and pathophysiological conditions. Therapeutic modulation of the CB2 presents a promising strategy for the treatment of several diseases like multiple myeloma, osteoporosis, pain etc. In the face of the huge therapeutic importance of the CB2, high resolution structural information and mechanistic details of receptor activation are poorly understood. This principally owes to the paucity of large amounts of purified recombinant functionally active CB2 *in-vitro.* GPCRs and most eukaryotic membrane proteins pose a formidable challenge for recombinant expression and purification. Limitations include low expression, toxicity towards host cells, loss of function etc. In an effort to produce functionally active recombinant CB2 that can be used for subsequent structural studies, in the present study, we have developed two distinct approaches for the functional expression and purification of CB2 from the *E. coli*.

In the first approach we used Mistic, an integral membrane protein expression enhancer, and TarCF, a C-terminal fragment of the bacterial chemosensory transducer Tar, as fusion partners at the N'- and C'-terminal respectively of the CB2 for its membrane targeted expression in the *E. coli* C43(DE3). Using the fusion partners individually or in combination, we found that CB2 fusion protein expression was maximal when both partners were used in combination. More

importantly, the fusion protein Mistic–CB2–TarCF localized to the *E. coli* membrane and these extracted membrane fractions exhibited functional binding activities with known CB2 ligands including CP55,940, WIN55,212-2 and SR144,528.

In the second approach, we expressed the CB2, in fusion with GST at its N'- terminal, as inactive inclusion bodies (IBs). The receptor protein was engineered to carry a 6 Histidine (His6) tag at its C'-terminal for subsequent immobilized metal affinity chromatographic (IMAC) purification. Pilot studies supported extraction of GST-CB2 in a denaturing detergent, *N*-Lauroyl sarcosinate (Sarkosyl) followed by exchange to Dodecyl-beta-D-Maltoside (DDM) for "on-column" cleavage. Post size exclusion chromatography, eluted purified monodisperse CB2 were subjected to refolding either in lipidic (DMPC) or proteic (Amphipol) environments. CB2 refolded in DMPC exhibited functional binding activities with known CB2 ligands including CP 55,940, SR144528 and PY2-64.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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ABBREVIATIONS

µm…… micrometer
µg……..…. microgram
µl……..….. microliter
2-AG…….…..... 2-arachidonoylglycerol
3D……..…… Three Dimensional
5-HT……... 5-Hydroxy-tryptamine
A_{2A}……..…… Adenosine Receptor
AC……...….. Adenylate cyclase
AEA……..….. N-arachidonoylethanolamide
AMP…....……Adenosine mono phosphate
ATP…....…..... Adenosine tri phosphate
BCA……..….. Bicinchoninic acid assay
β2-AR…..…… β2-Adrenergic Receptor
BLT1/2……. Leukotriene receptors 1 or 2
BSA……..……Bovine Serum Albumin
C'……...…..Carboxy terminal
cAMP….....cyclic Adenosine mono phosphate
CB1/2…….....Cannabinoid receptor 1 or 2
CBB……..... Coommassie Brilliant Blue
CDB……..…...Compound Dilution Buffer
cDNA…….....complementary DNA
CNS……..……Central Nervous System
CPM……..….. Counts Per Minute
CSFV…….....classical swine fever virus

CV……...…Column Volume

DDM…….…… Dodecyl-beta-D-maltoside

DMPC…...…. 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine

DNA…....….. Deoxy-ribonucleic acid

DOC……..…. Deoxycholic Acid

DPC……..….. n-dodecyl phosphocholine

DTT……..….. Dithiothreietol

E.coli …...…. *Escherichia coli*

EDTA…….…..... Ethylene diamine tetraacetic acid

EGTA…...…...Ethylene glycol tetraacetic acid

EL…….…..... Extracellular loop

fmol…….…... femtomolar

Fwd…..…..….. forward

g…….…..…..... gram

x g……..……..… standard accelaration due to gravity

GEF……..…GTP exchange factor

GHSR…….…..... growth hormone secretagogue receptor

GIRK……..….G protein regulated inward rectifying potassium channel

GPCR….....G protein coupled receptor

GTP…….....Guanosine 5'-triphosphate

Gα/βγ…….…. G protein (Gα and Gβγ subunits)

$G\alpha_i$…….…….....G alpha subunit inhibitory

$G\alpha_s$…...….....G alpha subunit stimulatory

HA…….……..… Hemagglutinin

HDM…….…..… hexadecyl-β-D-maltoside

His…….……..… Histidine

hrs…….……..… Hours

HTS……...… High-throughput screening

huPTHR1….. human Parathyroid Hormone Receptor 1

IB…..…….…. Inclusion Body

IL…..……...… Intracellular loop

IMAC……..... Immobilized Metal Affinity Chromatography

IMP…..…… Integral Membrane Protein

IPTG…...…… Isopropyl β-D-1-thiogalactopyranoside

K_d……...…… Dissociation constant

kDa…….…..... Kilo Dalton

K_i…….…… Binding Affinity of the inhibitor

KSI……..... Ketosteroid isomerase

L…….…..... Liter

LB…….. Luria Broth

LDAO.…… Lauryldimethylamine-N-oxide

LRTK…..….....leucocyte receptor tyrosine kinase

MAPK…..…… Mitogen-activated protein kinase

MBP…….…... Maltose Binding Protein

mins……..…... minutes

Mis……..… Mistic

ml…… milliliter

mM.....millimolar

mRNA…..…… messenger RNA

muCB1……. mouse Cannabinoid Receptor 1

N'……...…. Amino terminal

nM……..…..... nanomolar

NMR…….. Nuclear Magnetic Resonance

^{o}C……...……. degree Celcius

OD…….. Optical Density

PAGE……. Polyacrylamide Gel Electrophoresis

PE…..…….. Phosphatidyl Ethanolamine

pg…..…….picogram

PI3K…… Phosphoinositide 3-kinase

PIC…..…… Protease Inhibitor Cocktail

PMSF…….Phenylmethanesulfonyl fluoride

POPC/G…..… 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho choline / glycerol

PS…….. Phosphatidyl serine

Rev……... Reverse

RNA…..…..…Ribonucleic acid

rpm……...….. revolutions per minute

RT……..….room temperature

S.E.M.....Standard Error of Mean

SDS…….…....Sodium dodecyl sulfate

TarCF…....….Bacterial Chemosensory Aspartate Receptor (C terminal fragment)

TCA……..….. Trichloroacetic Acid

TCEP.....*tris*(2-carboxyethyl)phosphine

TEV…….....…Tobacco Etch Virus

TM……...….. Transmembrane

tPA……....….tissue Plasminogen Activator

Tris…….… tris(hydroxymethyl)aminomethane

Tris-HCl…..… tris - hydrogen chloride

tRNA…….…. transfer RNA

Trp…….…. Tryptophan

TrxA…..…..…Thioredoxin

Tyr……..…….Tyrosine

U…….…..…...Enzyme Unit

w/v…….…..… weight per volume

1.0 INTRODUCTION

1.1 RECENT ADVANCES IN EXPRESSION AND PURIFICATION OF GPCRS

1.1.1 The GPCR Superfamily

G-protein coupled receptors (GPCRs) comprise one of the largest superfamily of human receptor proteins which act as signal transducers. These receptors initiate internal signaling events in response to huge array of external stimuli known as “signals”. About 4% of the protein coding genome code for these receptors in humans (Bjarnadottir et al., 2006). Overall, GPCRs are classified into 6 groups based on their structural and functional similarities (Attwood and Findlay, 1994; Foord et al., 2005; Kolakowski, 1994). These are: Class A – Rhodopsin-like, Class B – Secretin receptor family, Class C- Metabotropic glutamate, Class D- Fungal mating pheromone, Class E- Cyclic AMP receptors and Class F- Frizzled/Smoothened (Figure 1.1). Amongst these, the Rhodopsin-like (class A) family of GPCRs comprises nearly 85% of the entire family of GPCRs and is further subdivided into 19 subgroups A1 through A19 (Dorsam and Gutkind, 2007). GPCRs respond to a variety of signals which include hormones, growth factors and endogenous ligands (Gaidamovich et al., 1978). Due to the huge diversity of activating ligands, GPCRs play a diverse array of roles in various physiological processes that include regulation of vision, smell, mood, behavior, immune modulation and the maintenance of

several other homeostatic processes (Kang and Koo, 2012; Sumiyoshi et al., 2013; Wasik et al., 2011; Zhou et al., 2012). Despite their functional diversity, all GPCRs share a common structural motif which is characterized by an extracellular amino (N') terminal domain, seven transmembrane domains (TM 1-7) and an intracellular carboxy (C')-terminal domain. The transmembrane domains are connected by three extracellular (EL 1-3) and three intracellular (IL 1-3) loops. The tertiary structures of these receptors are arranged as a barrel with a central core region which generally houses the ligand binding site. In cases where the ligand is a protein or a large peptide hormone, the extracellular loops 1 and 2 serve as ligand binding sites. The N'- and the C'-terminals play independent roles in ligand recognition and post-translational modification respectively (Kawamura et al., 1989; Kristiansen, 2004). Upon ligand binding and activation, the receptors undergo conformational change in the transmembrane region. These rearrangements are facilitated by the disruption of the ionic linkage and the toggle of the conserved tryptophan residue within the transmembrane domains of Rhodopsin family of GPCRs (Kobilka and Deupi, 2007). The movements within the transmembrane region allow the exposure of the cognate G protein binding site which leads to binding of the G protein and subsequent signal transduction (Trzaskowski et al., 2012).Recent studies suggest that GPCRs have their own intrinsic degree of activity independent of ligand binding, known as basal constitutive activity (Kobilka and Deupi, 2007). However the presence of activating ligands results in formation of “activated GPCR” which is capable of G protein binding. G proteins are made up of Gα and Gβγ domains. The Gα subunit houses the GTP exchange factor (GEF) region of the G protein. Post ligand binding the GEF in an activated G protein recruits a GTP leading to the dissociation of the Gα and the Gβγ subunits, which independently have limited downstream activation properties. The subsequent role of the individual Gα subunit depends on the cognate GPCR which also determines the

downstream signaling pathway. The stimulatory Gα unit ($G\alpha_s$) activates the cellular protein adenylate cyclase and lead to increase in cyclic AMP (cAMP) levels while the $G\alpha_i$ (inhibitory) leads to the inhibition of the adenylase cyclase activity resulting in reduction of cAMP levels in the cell (Simonds, 1999). The activated Gβγ moiety has independent signal transduction capabilities and acts particularly on different ion channels e.g. G protein regulated inward rectifying ion channels (GIRKs) and N-type voltage gated Ca^{2+} channels (Wickman and Clapham, 1995). Downstream signaling events following GPCR activation are complicated and are extremely diverse due to the presence of a large array of secondary effectors which are specific to the GPCR.

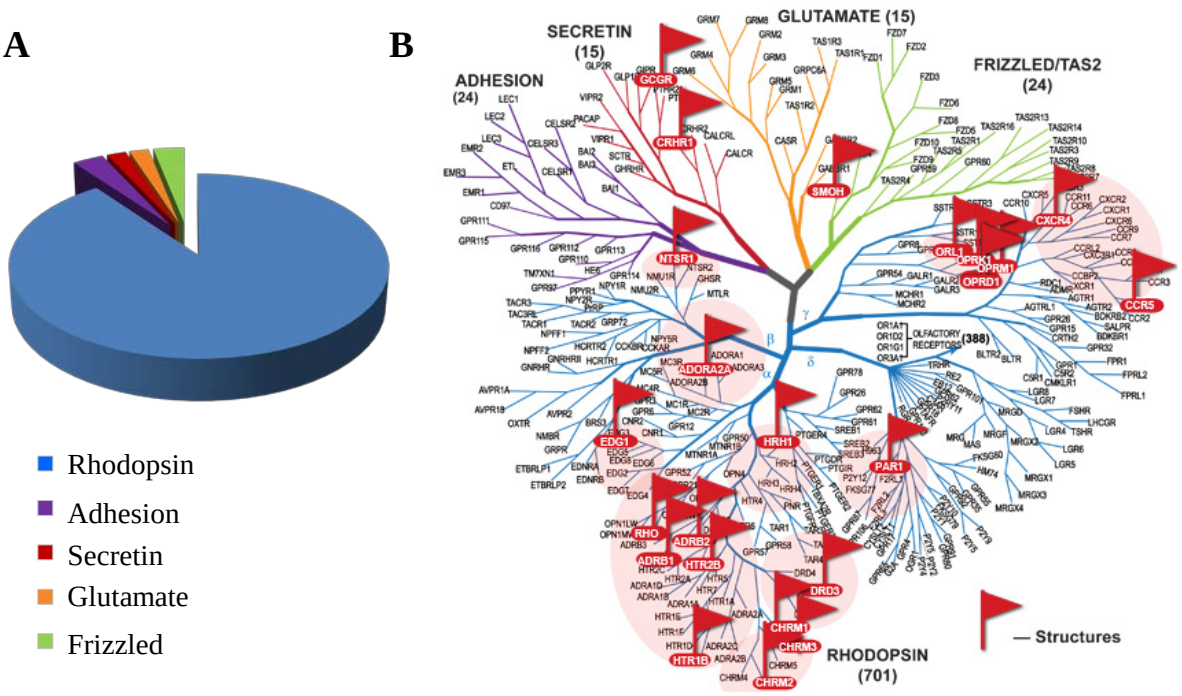


Figure 1.1. The organization of GPCR superfamily and known structures

(A) The GPCR superfamily is organized into five subfamilies based on their sequential similarities. The majority of the receptor proteins belong to the Rhodopsin family GPCRs . (B) Family tree of the entire GPCR superfamily showing the number and position of the GPCRs with known structure.

1.1.2 Necessity and challenges in the expression and purification of GPCRs

GPCRs altogether make up one of the largest class of drug targets. Due to their significant roles in various physiological processes they are widely implicated in a large number of pathophysiological conditions as mentioned in the previous section. It has now also been established that they are involved in the growth and metastasis of several different types of tumors. Substantial amount of research both in the industry and the academia is focused towards understanding the mechanism of action of these receptors. Alongside, a closely related focus of most researchers is the design and development of more selective and potent ligands for modulating the action of these receptors for the desired therapeutic effect(s). Despite the huge therapeutic implications of the receptors, the high resolution structure of the receptor is available for only twenty two out of eight hundred GPCRs (Venkatakrishnan et al., 2013) (Figure 1.1). This lag in the number of high resolution structure or mechanistic details of activation process creates a formidable challenge in GPCR drug discovery, particularly in structure based drug design. Several other computational or ligand based drug discovery methods have been developed to work around this barrier. These methods rely on screening large ligand libraries *in-silico*. However, generated lead molecules may suffer from the lack of potency and specificity. Lack of specificity is a huge problem when different subtypes of the same receptor are present and have diverse physiological roles, for example the Cannabinoid Receptors 1 and 2 (CB1 and CB2) which will be discussed in much greater detail in Section 1.3.

The challenges in obtaining high resolution X-ray crystal or Nuclear Magnetic Resonance (NMR) structure of a GPCR are manifold. Due to the transmembrane structure of the GPCRs and the inefficient membrane insertion process the over-expression of the GPCR in prokaryotic expression hosts prove toxic. The yield of recombinant GPCR is comparatively much lower than

the yield of globular or soluble proteins in an expression host. Extraction, enrichment and purification of the GPCRs require the presence of detergents which may negatively affect the GPCR yield and stability. These detergents in residual amounts may also deter the formation and growth of crystals. However certain amphipathic detergents may be well tolerated during crystal growth in combination with lipid or lipid-like molecules. Finally, GPCRs have very less area for the formation of crystal contacts. The presence of the intracellular loop3 and other regions of inherent flexibility are likely impede or may totally prevent the growth and formation of crystals. Due to the presence of several roadblocks towards high resolution structure of GPCRs, optimization of several parameters at each step of the process is required (Figure 1.2).

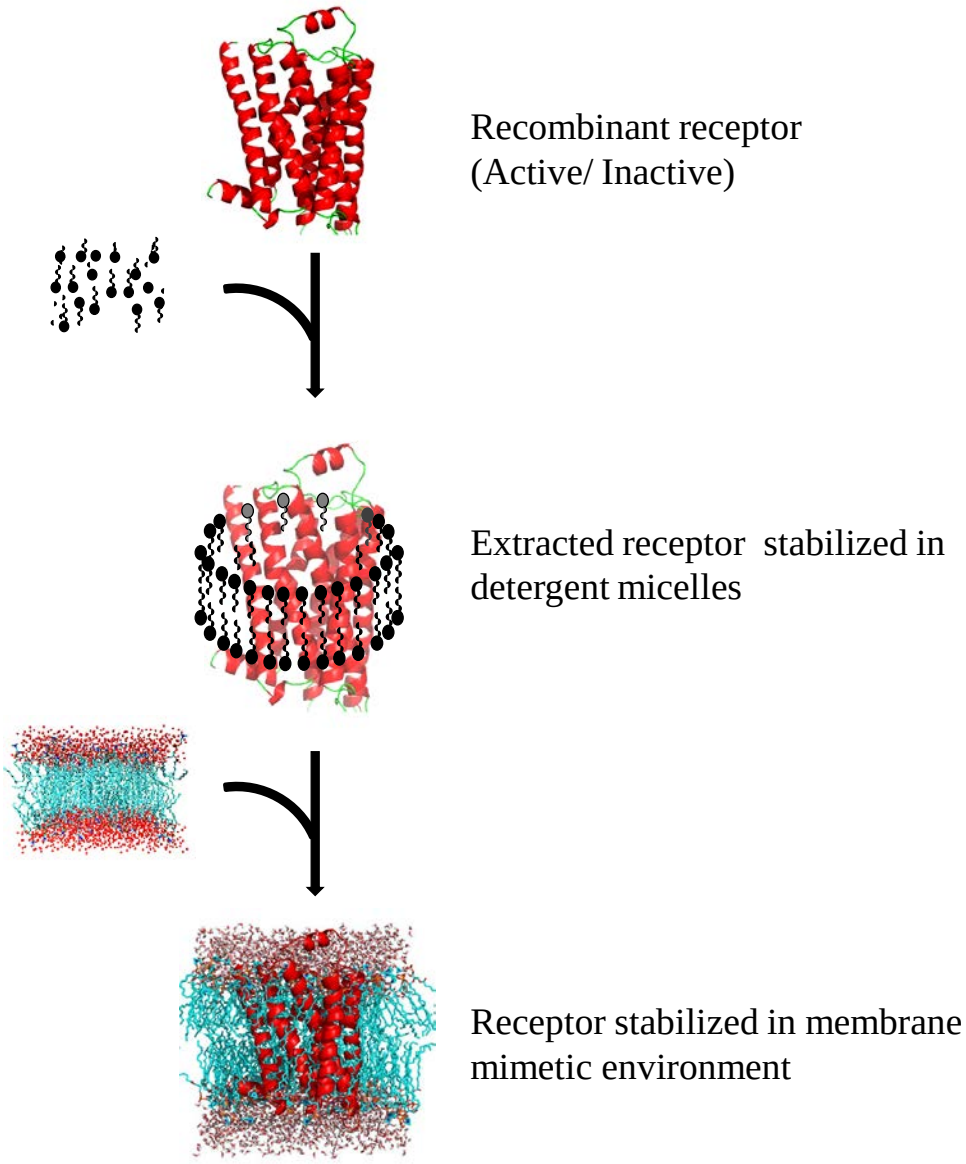


Figure 1.2. Challenges towards generation of stabilized GPCR *in-vitro*.

Recombinant receptor production is toxic for the host organism. The produced receptor requires detergent for extraction and solubilization in the buffer which can then be subjected to chromatographic purification. Finally the detergent solubilized receptor requires to be exchanged to the stabilizing environment to obtain the receptor in the correct / native structural disposition.

1.1.3 Current methods and developments for GPCR expression and purification

The challenging nature of membrane protein GPCR expression has led to the trial and development of optimized processes for the expression of GPCRs. Several hosts such as bacteria *H. salinarium, L. lactis* ,several other species of yeasts like *S. cerevesiae* and *S. pombe* and insect cells like *Sf9* and *Hi5* have also been used for GPCR expression.

Expression of the receptor protein in the *E.coli* results in the formation of insoluble aggregates known as inclusion bodies (IBs) (Bane et al., 2007). Previous studies in our lab were directed towards structural studies of individual CB2 transmembrane helices (Xie et al., 2004; Zhang and Xie, 2008; Zheng et al., 2005). Expression of eukaryotic membrane protein has been achieved in the *E. coli* by designing and expressing fusion proteins which carry bacterial membrane proteins in either or both terminals of the protein of interest. This method was pioneered by Grisshamer et. al has been applied for many GPCRs (Grisshammer, 2009; Grisshammer et al., 1993; Grisshammer et al., 1994; Grisshammer and Tate, 1995) including the studies of Yeliseev et. al on the CB2 receptor (Berger et al., 2010; Krepkiy et al., 2007; Krepkiy et al., 2006; Yeliseev et al., 2005). Many further modifications of this method were made and newer fusion partners discovered and validated including our previously reported study (Chowdhury et al., 2012). Functional GPCR production and purification was achieved with varying degree of success from these constructs however it is challenging to maintain the structural disposition of the receptor while extraction, chromatographic steps, tag removal etc.

Baculovirus infected *Spodoptera frugiperda* (Sf9) cells have successfully led to the production of GPCRs yielding crystal structures (Aloia et al., 2009). *Sf9* cells have the advantage of a eukaryotic transcription and translation machinery and a eukaryotic membrane composition which favors eukaryotic GPCR expression, membrane translocation and functional

activity (Aloia et al., 2009). However, crystal formation in GPCRs requires stabilization of the inherently flexible intracellular loop 3 (IL3) region by the more stable and crystallizable T4Lysozyme or stabilized by the presence of Anti-IL3 antibody Fab fragments (Rasmussen et al., 2011; Rasmussen et al., 2007; Rosenbaum et al., 2007; Rosenbaum et al., 2009; Rosenbaum et al., 2011). The procedure to generate the GPCR chimera involves the critical adjustment to obtain the most stabilized receptor while still retaining its complete functional activity both in terms of ligand binding and signal transduction. Nevertheless crystal structure of several key members, particularly of the Rhodopsin family GPCRs, e.g. β2-Adrenergic Receptor, A2a Adenosine Receptor, Dopamine Receptor, Chemokine Receptor and more recently the Kappa –opoid Receptor etc were arrived at by this method (http://gpcr.scripps.edu/).

Procedure for membrane targeted GPCR expression in *E. coli* or *Sf9* requires the generation of functional chimeric GPCR. An entirely different approach is to express the GPCR in high amounts as inactive inclusion bodies (IBs) followed by purification and refolding to functionally active receptor (Baneres et al., 2011). One of the advantages of producing GPCRs, or for that matter, any heterologous gene product as IBs is that they mostly contain the recombinant protein in high purity (99%). However the IBs may also contain chaperones and membrane fragments. Secondly, the IBs are formed as tight clusters, this self association would allow masking the proteolytic sites hence the IBs are much more refractory to proteolytic cleavage. The IBs are usually also very stable mechanically and can be isolated and enriched from the cell preparations by centrifugation. For several examples e.g. mouse Cannabinoid Receptor 1(muCB1), human Parathyroid Hormone Receptor 1(huPTHR1)(Michalke et al., 2010), Chemokine receptor (Park et al., 2006)and more the yield of membrane protein receptors would increase more than thousand folds when expressed as inclusion bodies.

Table 1.1. Table illustrating the different expression systems for GPCRs and their associated characteristics.

Modified from (Lundstrom, 2005)

Expression System (host)		**Advantage**	**Disadvantage**	**Reference**
E. coli	Membrane targeted	Receptor is pharmacologically active	No post-translational modification. Fusion partners required	(Luca et al., 2003)
	Inclusion body	Much higher yields of the protein of interest	Receptor is not active, refolding required	(Baneres and Parello, 2003)
Pichia pastoris		Relatively easy. High cell biomass obtained	Clone selection difficult, cells very sturdy due to the presence of thick cell wall	(Weiss et al., 1998)
Baculovirus		Very similar to mammalian expression system	Viral stock production is slow and unstable.	(Mazina et al., 1994)
Mammalian	Transient	Native	Transfection efficiency dependent	(McAllister et al., 1992)
	Stable	Native and inducible. Long lasting production system	Long generation times, cell lines may not be stable	(Reeves et al., 2002)
Cell free translation		Simple and fast	Very low yield of recombinant protein, insertion to lipid bilayer is problematic	Mikako Shirozu, *unpublished data*

1.2 *E.COLI* AS A HOST FOR GPCR EXPRESSION AND RELATED METHODOLOGIES

1.2.1 Protein production in the *E.coli*

Escherichia coli are a gram negative, facultative anaerobic bacterium. This bacterium is commonly found in the lower part of the gastro-intestinal tract of warm blooded mammals. Most *E. coli* are harmless, while some may be the causative agent of food poisoning in humans. These rod shaped bacterium is ~ 2 µm long and has a diameter of ~ 0.5 µm (Kubitschek, 1990). The mean cell volume of the bacterium is ~0.6-0.7 cubic µm. The *E. coli* has traditionally been used as the workhorse for expression of wide variety of proteins by recombinant DNA technology. An explicit understanding of the process of transcription and translation in *E. coli* allows researchers to modulate several steps in the procedure of protein production (Lee, 1996; Russo, 2003). Commonly, the *E. coli* expression system uses the T7 RNA Polymerase system. One of the main disadvantages of this system is the high basal level of endogenous protein expression which consequently reduces the amount of the exogenous protein produced and the final yield of the protein of interest. The development of the T7/lac operon system allows suppression of the basal level expression of the protein of interest until the cells have grown to a particular stage in their growth curve. At this point, the cells can be induced with the chemical compound Isopropyl β-D-1-thiogalactopyranoside (IPTG) which is a chemical non-hydrolysable analogue of lactose and serves to induce expression of the exogenous protein of interest from its inducible promoter. *E.*

coli pLysS and pLysE strains carry the plasmid for the T7 lysozyme which is the inhibitor of the T7 polymerase. The addition of 0.5-1% glucose in the culture media acts as a catabolite repressor for a protein under the control of the lac operon (Moses and Prevost, 1966). The optimum growth temperature for the *E. coli* is 37°C. However a culture may be subjected to lower growth temperatures as low as ~20°C for the controlled production of recombinant proteins. The *E. coli* is one of the most versatile hosts and perhaps, plays one of the most important role in recombinant protein production. Some of the recent excellent developments in this field include production of the correctly folded and post-translationally modified proteins in *E. coli* like human plasma protein tissue plasminogen activator (tPA). This was achieved by engineering mutant strains of *E. coli* that maintain the cytoplasm in a much lesser reduced state and thus are impaired in the reduction of glutathione and thioredoxin (Bessette et al., 1999). Adding another feather to the cap is the production of the glycosylated proteins, which was achieved by engineering the N-linked glycosylation machinery from the bacteria *Campylobactor jejuni* (Wacker et al., 2002). These recent major breakthroughs combined with the traditional advantages of bacterial protein production still ranks the *E. coli* as a scientist's first choice of expression host for the production of recombinant proteins.

1.2.2 Advantages and Disadvantages for GPCR production in *E.coli*

The main advantages of using *E. coli* as a host for expressing recombinant proteins include their well-studied life cycle, short doubling time and its easy handling in the laboratory. Among other advantages are the low cost and ability to genetically modify the organism. However, one of the

major disadvantages of this expression system is the lack of post-translational modifications on expressed eukaryotic proteins. Another disadvantage is its inability to translocate proteins to their subcellular destinations, for example the transport of membrane proteins to the membrane. The development of a better and more capable *E.coli* strain is always one of the chief topics of research.

The biggest disadvantage of GPCR production in *E. coli* stems from the fact that bacteria do not express GPCRs and thus lack the machinery for synthesis and translocation of such membrane proteins. Moreover, their inability to modify eukaryotic proteins post translation adds on to the problem at hand. The most common post-translational modifications in GPCRs include glycosylation, phosphorylation and palmitoylation. Although the function of these modifications for a given GPCR cannot always be predicted, many GPCRs can function without these modifications. In applications like the formation of crystals, post-translational modifications are often removed from the recombinant protein provided; it can withstand the removal without complete or partial loss of function. This facet of *E. coli* expression represents an advantage over mammalian or cell based systems. The expressed GPCR with the help of fusion partners may be targeted to and inserted in the inner bacterial membrane. However, the lipid composition of the membrane also contributes to specific requirements by the receptor proteins to be targeted to the membrane. Bacterial inner membrane composition is significantly different from that of eukaryotic cells, in lacking cholesterol. Also the mammalian membrane is composed of much higher levels of phosphatidylserine (PS) which in bacteria is converted to phosphatidylethanolamine (PE) by the enzyme phosphatidylserine decarboxylase. Many GPCR known so far like the oxytocin receptor, human μ-opoid receptor and the dopamine D1 receptor require specific lipid component for optimal functioning (Opekarova and Tanner, 2003).

However, in-spite of having disadvantages and differences from the mammalian expression system, the *E. coli* has quite successfully been used for the production of many functionally active GPCRs. Yet, it seems to be difficult to generalize rules for the protein engineering and setting up expression conditions. Expression levels, even to the extent of all or none, vary between receptors which belong to the same GPCR family. Besides, as the mechanisms of action of fusion partners are unclear the choice and use of particular fusion partner is a matter of hit and trial. Table 1 below summarizes the comparison of advantages and disadvantages of GPCR expression in the *E.coli* versus baculovirus infected insect cell lines.

Table 1.2 **Comparing GPCR expression in Sf9 cells vs *E. coli***

Attribute	**GPCR obtained for crystal structures**	**Obtained from *E. coli***
Easy, fast, cheap	Costly time taking insect cell growth	Easy, rapid and cheap
Native structure	GPCR modified by removing terminals, inserting T4 lys	GPCR modification not required
Isotopic labelling	Not possible	Easily possible
Dynamic studies	Rigid stabilized structure	Can be conducted

1.2.3 Current methods and developments of GPCR expression and purification from *E. coli*

GPCR production in the *E. coli* has developed and evolved with the advent of newer techniques which support either membrane targeted or inclusion body directed GPCR expression. These methods have their own advantages and disadvantages. The methods can be divided into expression of either 'functional modified GPCR' or of 'non-functional, unmodified GPCR' in the *E. coli*.

Functionality can be preserved by the membrane targeted expression of the GPCR. The fusion of the *E. coli* periplasmic protein, MBP with the N' terminal of a GPCR results in its translocation to the bacterial periplasmic membrane post translation. Several studies have reportedly used this approach and successfully conducted the membrane targeted expression of GPCRs. These include the rat neurotensin receptor, rat neurokinin 2 receptor, human adenosine A2A receptor, human 5HT1a receptor, M1 and M2 muscarinic acetylcholine receptor, human β2a receptor, and the human cannabinoid CB2 receptor (Grisshammer, 2009; Weiss and Grisshammer, 2002b; Yeliseev et al., 2005). Newer direction towards the use of alternative fusion partners like Mistic, an integral membrane protein expression enhancer, and TarCF, a C-terminal fragment of the bacterial chemosensory transducer Tar showcases the continued developments in this field (Chowdhury et al., 2012). A major setback in this approach is that the functional GPCRs within the bacterial periplasmic or inner membrane may lose their functional activity upon isolation from the membrane. They may also have altered structural disposition when inserted to artificial membrane mimetic environments. Also recombinant GPCR in *E. coli* membrane may display lesser affinity for its ligands when compared to it being expressed in an eukaryotic membrane milieu like transfected mammalian cell lines.

A diametrically opposite approach is the expression of ‘non-functional, unmodified GPCR’. In this case GPCR is first expressed as inclusion bodies and then refolded *in-vitro* to its functionally active state. This approach depends on the isolation and purification of the GPCR under denaturing conditions and then gradually transferring it to milder detergents and membrane mimetic environment for structural refolding (Baneres et al., 2011). Several GPCRs have been refolded to complete or partial functionality by this approach (Baneres et al., 2003; Baneres et al., 2005; Kiefer et al., 1996). However this method is not straightforward and requires optimization and trial of every step beginning from expression to purification and most importantly refolding to functionality.

1.3 CANNABINOID RECEPTOR 2

1.3.1 The Endocannabinoid System

The endocannabinoid system includes group of endogenous lipids, enzymes that synthesize and degrade such lipids and their cognate receptors (Pertwee, 2006). This system controls in overall various physiological processes like mood regulation, immune modulation, pain, memory (Fortin and Levine, 2007). The endogenous lipids that make up the endocannabinoid system include anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG). Enzymes of the endocannabinoid system include fatty acid amide hydrolase and the monoacylglecerol lipase. The Cannabinoid Receptor 1 (CB1) and the Cannabinoid Receptor 2 (CB2) make up the receptors of the endocannabinoid system. The binding of endo- or exogenous cannabinoids to their cognate receptors (CB1 and CB2) in the CNS and the periphery respectively, lead to inhibition of the enzyme adenylase cyclase thereby triggering further intracellular downstream signaling. The CB1 and CB2 receptors belong to the Rhodopsin family of GPCRs. In the CNS, several endocannabinoids (e.g. 2-AG) are synthesized as a secondary effect of the neurotransmitters on the post-synaptic neuron. The effects of these cannabinoids are exerted through the CB1 receptor and involve memory, mood regulation, behavior, appetite, etc (Hampson and Deadwyler, 1999; Pertwee, 2001). Binding of a cognate ligand to the CB2 receptor results in immune modulation. In summary, the endocannabinoid system comprises and maintains a complicated set of events, interactions and outcomes which regulate diverse physiological processes.

1.3.2 CB2 Receptor Structure

The high resolution three dimension (3D) structure of the CB2 receptor has not been resolved, as of date. Efforts to purify reconstitute and obtain high resolution structural information of the receptor and several attempts to generate the 3D structure of the CB2 by homology modeling or *ab-initio* calculations have been reported.

The CB2 receptor follows the general structural pattern of Rhodopsin family GPCRs and has a glycosylated N'-terminal, seven pass transmembrane domains and an intracellular C'-terminal tail (Cabral and Griffin-Thomas, 2009; Galiegue et al., 1995a). Overall the CB1 and CB2 shares a sequence homology of ~44% based on amino acid composition and the degree of similarity in the transmembrane region is ~68% (Cabral and Griffin-Thomas, 2009; Munro et al., 1993). In an effort to get a general idea of the receptor structure, homology models of the receptor were generated by Xie *et. al* (Xie et al., 2003) and Montero *et. al* (Montero et al., 2005). However, both these studies lacked an adequate number of templates and were constructed only on the basis of the structure of the bovine rhodopsin (Palczewski et al., 2000). The receptor model arrived at by Xie *et. al* shows that CB2 consists of structural features typical to Rhodopsin family GPCRs. The seven helices are tilted at various angles relative to the plane of the membrane with an extensive network of hydrogen bonding among the residues of the transmembrane helices. The presence of the conserved D(E)RY motif and the salt bridge interaction of the residue Arg 131 (TM3) with the residue Asp 260 (TM6) was observed in the model. This salt bridge interaction is the conserved feature of all Rhodopsin family receptors. Even though the 3D structure of the CB2 has not been solved yet, a huge impetus to homology modeling has been provided by elucidation of the structures of the several other more closely

related GPCRs which provide many more templates for more in-depth and higher confidence model building.

Several mutagenesis studies have also been performed which indicate the presence of ligand binding and/or stabilizing roles of the various key domains of the CB2 receptor. For example, in a study by Zhang *et.al* the conserved residue Trp 194 was found to mediate the process of conformational rearrangement during receptor activation and G protein binding (Zhang et al., 2011). Although these studies have provided a huge impetus for CB2 structure function research and drug discovery, it is crucial to work towards and determine the high resolution structure of the CB2 receptor.

1.3.3 CB2 Receptor Expression Profile

The CB2 receptor was cloned and discovered in 1993 by Munro *et. al* and was initially reported to be expressed in macrophages and to marginal levels in the spleen (Munro et al., 1993). The gene for the study was cloned from the complimentary DNA (cDNA) of the human promyelocytic leukemia line HL60. The cloned DNA was transfected to expression cell lines and membrane preparations were used to determine the presence of receptor by testing binding with known Cannabinoid ligands. The expression profile of the CB2 receptor is currently well established. The presence of the CB2 in immune cells was initially reported by Galiegue *et. al* (Galiegue et al., 1995a). The descending rank order of level of CB2 expression in the immune cells follows: B-cells > natural killer cells >> monocytes > polymorphonuclear neutrophil cells > T8 cells > T4 cells. CB2 receptor is also expressed in the gastrointestinal system where they mediate the process of intestinal immune response. CB2 agonists are routinely used for the treatment of several inflammatory diseases like Crohn's disease and ulcerative colitis (Capasso et

al., 2008; Wright et al., 2008). CB2 is also expressed in mast cells where they may mediate inflammatory response (Elphick and Egertova, 2001). The Cannabinoid mediated modulation of CB2 in mast cells are thought to decrease the noxious stimuli. The mRNA content of the CB2 in the spleen is comparable to that of the CB1 in the brain. The respective expression of the CB1 and the CB2 in the CNS and periphery were believed to be like water tight compartments until the discovery of the fact that trace amounts of the CB2 receptor is also found in the brain (Onaivi, 2006). However unlike the CB1, the CB2 is mainly expressed in the supporting cells of the CNS like the microglia and not in neurons (Cabral et al., 2008; Pertwee, 2006).

1.3.4 Physiological role and therapeutic potential of the CB2 Receptor

One of the most significant roles of the CB2 receptor is probably in the modulation of immunological activities and inflammatory responses in leukocytes (Kaminski, 1998). Cannabinoid compounds disrupt leukocyte function by markedly inhibiting the action of adenylate cyclase, protein kinase A and decreased DNA binding of the cAMP response element binding proteins. These and related effects of the cAMP signaling pathways modulate the expression and secretion of cytokines which have deep impact on the immune system (Kaminski, 1996). Recent studies on the Cannabinoid agonist JWH-015 has revealed that within T cells changes in the cAMP levels lead to the phosphorylation of leucocyte receptor tyrosine kinase (LRTK) at the conserved Tyr505 which leads to inhibition of T cell receptor signalling. Studies are ongoing to determine and use the effects of the Cannabinoid ligands for the treatment of pain, particularly neuropathic pain (Cheng and Hitchcock, 2007). The involvement of the CB2 receptor in pain can be corroborated with the expression of the CB2 in the spinal cord and the

dorsal root ganglion of neuropathic pain animal models (Pertwee, 2008). CB2 receptors also alter the maintenance and homing of B cells. These and several more findings clearly indicate the role and scope of modulating the CB2 for the treatment of several pathogen related or autoimmune disorders.

The presence of the CB2 in brain microglia has demonstrated possible therapeutic application for the treatment of Alzheimer's disease. The CB2 agonist JWH 015 leads to activation of macrophages and removal of beta amyloid plaques (Tolon et al., 2009). The accumulation of the plaques leads to the subsequent neurodegeneration (Tiraboschi et al., 2004). Overall the CB2 receptor stands as a very promising therapeutic target for several significant pathological conditions.

1.3.5 Overall goal and approaches towards production of functionally active CB2 Receptor

In summary, the Cannabinoid Receptor 2 is a very significant therapeutic target (Basu and Dittel, 2011; Patel et al., 2010). Continued research is uncovering newer roles for the receptor in homeostasis and diseases. The lack of selective modulators in the market that can target the CB2 receptor necessitates CB2 drug discovery efforts. Candidate compounds often either have low efficiency or specificity and can target and activate the closely related CB1 receptor which then can lead to undesirable effects. High resolution structural information for CB2 will facilitate the understanding of the molecular basis of ligand functionality. This will then allow for Structure Based Design of potent and selective CB2 receptor modulators. However as discussed in Section 1.1.2, the challenges towards structure elucidation of CB2, or any GPCR for that matter, arises from the lack of purified and functionally active receptor. Thus in this project, our goal is to

develop a method for producing purified, functionally active CB2 receptor within membrane mimetic environments (Figure 1.3). For this purpose, we chose to use the *E. coli* expression system as it is the fastest, cheapest expression host that allows for the easy production of isotopically labeled protein. This protein can then be used by NMR and EPR methods to study CB2 dynamics with or without ligand activation. The developed methodology and the available receptor will then open up newer research both in GPCR expression purification and structural or biophysical characterization of the CB2 receptor respectively. To achieve this goal, we plan to carry out CB2 receptor expression, purification by two different approaches (Figure 1.3). In the first approach, CB2 will be produced as fusion protein with novel fusion partners Mistic and TarCF. In the second approach CB2 will be produced as inactive inclusion bodies which will then be subjected to refolding trails using different stabilizers or membrane mimetic environments to generate functionally active CB2 receptor *in vitro*.

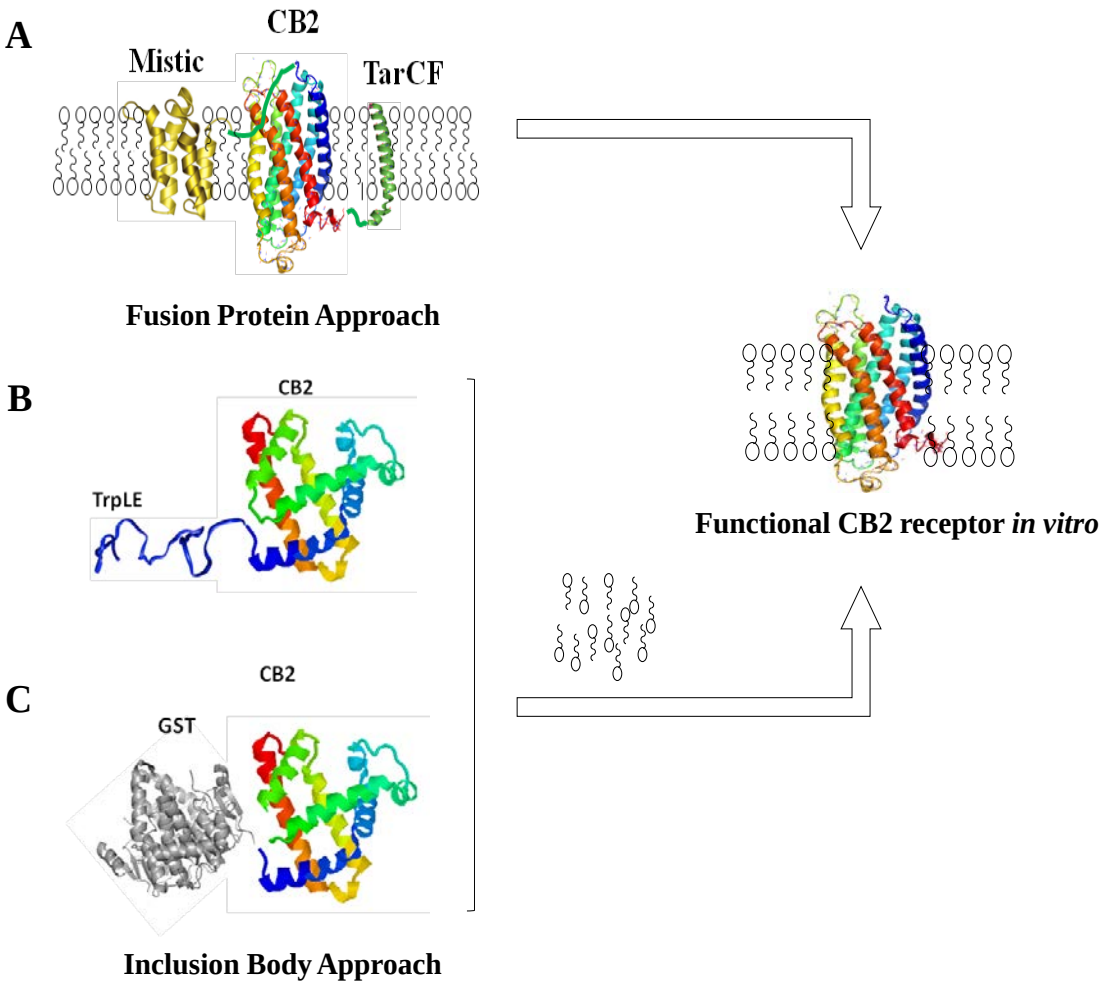


Figure 1.3. Overall strategic approach for the generation of functionally active CB2 receptor *in-vitro.*

The overall goal of the project is the generation of functional CB2 receptor *in-vitro*. To carry out CB2 functional expression in the *E. coli* inner membrane CB2 will be expressed with fusion partners Mistic and TarCF as N and C terminal fusion partners respectively (A). (B) & (C) Inclusion body directed approach using the TrpΔLE and the GST tag partners.

2.0 EXPRESSION, OPTIMIZATION AND CHARACTERIZATION OF THE CANNABINOID RECEPTOR 2 WITH FUSION PARTNERS IN THE *E. COLI*

2.1 INTRODUCTION

2.1.1 Therapeutic significance of CB2 Receptor

The physiological effects of endogenous and synthetic cannabinoid ligands are mediated by two cell surface G-protein coupled receptors cannabinoid receptor subtype 1 (CB1), expressed abundantly in the brain and subtype 2 (CB2), expressed mainly in the immune system (Attwood and Findlay, 1994). These two receptors, share 68% sequence homology in their transmembrane domains and 44% similarity in their overall receptor sequences (Galiegue et al., 1995b; Howlett et al., 2002; Munro et al., 1993; Xie et al., 2003).

After stimulation, the CB2 receptor couples to Gαi to negatively regulate cyclic AMP levels by inhibiting adenylase cyclase activity (Bayewitch et al., 1995; Gonsiorek et al., 2000), and to the Gβγ domain to enhance MAPK and PI3K activation, ceramide production and downstream gene expression (Bouaboula et al., 1999a; Bouaboula et al., 1999b; Bouaboula et al., 1996). Clinically, modulation of the CB2 signaling exhibits great potential for the treatment of inflammatory and autoimmune diseases, cancer, heart and bone disorders as well as neurodegenerative disorders (Alexander et al., 2009; Lozano-Ondoua et al., 2010; Martin-

Moreno et al., 2011; Pacher and Mechoulam, 2011; Zajicek and Apostu, 2011). In addition, CB2 activation has also shown to have neuroprotective and analgesic effects in animals via unclear mechanisms (Anand et al., 2009; Cabral et al., 2008). CB1 is highly expressed in the brain and therapeutic modulations of this receptor have resulted in adverse psychotropic side effects (Cahill and Ussher, 2007; Kelly et al., 2011). Selective modulation of CB2, however, would be able to achieve the desired therapeutic effect without such psychotropic side effects due to no or very low expression of CB2 in the central nervous system (CNS). Therefore, the CB2 receptor is a significant and desirable target for therapeutic intervention requiring more in-depth information regarding the receptor structure and function to design highly selective ligands. However, expression levels of CB2 are very low in native tissues, and structure determination of CB2 has been impeded due to the inability to produce sufficient amounts of the receptor proteins with high homogeneity and natural ligand binding activity.

2.1.2 Efforts towards heterologous GPCR Expression

Different hosts have been employed to improve the expression levels of GPCRs. Baculovirus-infected insect cell lines have been used to produce GPCRs including the cannabinoid receptor 2 (Nowell et al., 1998), beta 2-adrenergic receptor (Rasmussen et al., 2011; Rasmussen et al., 2007; Sarramegna et al., 2003b) , chemokine receptor (Kwong et al., 1998) and the A2a adenosine receptor (Cherezov et al., 2007; Jaakola et al., 2008); most of which have been structurally modified to facilitate receptor stability and crystallization. Yeast cells also provide eukaryotic environment for post-translational modification of the exogenous GPCRs (Kim et al., 2005; Naider et al., 2004). However, compared to mammalian cells, they differ in membrane

composition and posttranslational modification (Sarramegna et al., 2003a). While lacking post-translational modifications, the bacterial system offers several unbeatable advantages for the expression of exogenous proteins: fast, homogeneity in protein production, low cost and ability to isotopically label the protein of interest for subsequent NMR studies (Hockney, 1994). Previously, *E. coli* was used in our lab to express CB2 receptor fragments by directing the fragment expression to inclusion bodies using the TrpΔLE leader sequence (Xie et al., 2004; Zheng et al., 2005). The CB2 receptor fragment produced in *E. coli* and reconstituted in Brij 58 showed > 75% preservation of the alpha helical structure (Zhang and Xie, 2008). However, the methodology developed in these studies may not be applied to the intact receptor without substantial modifications.

2.1.3 Fusion partners for the functional expression of GPCRs in *E. coli*

For heterologous expression of eukaryotic membrane proteins, fusion protein technology in *E. coli* has been successfully applied for numerous proteins (Table 2). For the integral membrane protein, neurotensin receptor, the expression level for this receptor was enhanced 40-fold when neurotensin was fused to maltose binding protein (MBP) at the N'-terminus and the signal peptide sequence Endotoxin B at the C'-terminus (Grisshammer et al., 1993). Related methodologies have also been used for the production of the rat neurokinin A receptor (Grisshammer et al., 1994) and human adenosine A2a receptor (Weiss and Grisshammer, 2002a). In addition, expression of the CB2 receptor by using MBP as an N'-terminal fusion partner and Thioredoxin as a C'-terminal fusion partner has also been reported (Berger et al., 2010; Krepkiy et al., 2007; Yeliseev et al., 2005).

Table 2.1 Fusion tags used for Affinity purification and solubilization of recombinant proteins

Adapted from (Arnau et al., 2011)

Name of Tag	Length of tag (aa)	Usage conditions
Chitin-binding domain	52	Can bind to insoluble chitin only
c-myc	10	Purification based on monoclonal antibodies
Elastin-like peptides	18 - 320	Change in temperature can aggregate protein : tag removed with intein
FLAG	8	Purification is Ca^{2+} dependent and based on mAb
Glutathione S-transferase	201	Glutathione or GST antibody affinity
HA-tag	9	Hemagglutinin from Influenza virus, antibody-based purification
His-tag	5-15	Native or denaturing conditions for purification
Maltose binding protein	396	Affinity to Amylose
NusA	495	Increased solubility in *E. coli*. Affinity tag needed for purification
Softag1, Softag 3	13, 8	mAb responsive to Polyol
S-tag	15	Affinity to S-protein resin
Streptag II	8	Modified streptavidin; eluted with biotin analog
T7-tag	11-16	Purification based on monoclonal antibodies
Thioredoxin	109	Affinity to modified resin
Xylanase 10A	163	Cellulose based capture; glucose elution

Determining the correct fusion partner(s) to optimize GPCR expression is not empirical but largely depends on the receptor in question. We have used two new fusion partners Mistic and TarCF for CB2 expression. Mistic is an unusual *B. subtilis* membrane protein (Kefala et al., 2007; Roosild et al., 2005); TarCF is the C'-terminal fragment of bacterial aspartate chemosensory transducer Tar (Antommattei et al., 2004; Krikos et al., 1985). While Mistic and TarCF have been routinely used as fusion partners to enhance expression and stabilization of proteins, their effects on the expression and stabilization of GPCRs remain obscure and unexplored. In the present study, we have evaluated the roles of several fusion partners including Mistic, TarCF and TrxA, alone or in combination, to drive the functional expression of the CB2 receptor in *E. coli* . To facilitate the fusion protein release and purification, enzymes are used routinely (Table 3). Factor Xa/TEV sequences and multi-His tags were introduced into our expression construct, Mis-CB2-TarCF. Culture conditions were optimized to determine the conditions for maximum fusion protein yield.

Table 2.2. **Enzyme based methods to remove fusion tags**

Adapted from (Arnau et al., 2011).

Enzyme	Cleavage site	Comments
3C protease	ETLFQ*GP	Protease removed by Ni-chelating or GSH resin
Aeromonas aminopeptidase	Exopeptidase	Cleaves at N-terminal sequentially; Zn^{2+} required for activity; cannot cleave E, D, X-P; acts on M, L
Aminopeptidase M	Exopeptidase	Cleaves at N-terminal, cannot cleave X-P
Carboxypeptidase A	Exopeptidase	Cleaves at C-terminal. No cleavage at X-R, P
Carboxypeptidase B	Exopeptidase	Cleaves at C-terminal; acts on basic amino acids (R, K)
DAPase (TAGZyme)	Exo(di)peptidase	Cleaves N-terminal; Carries C-terminal His tag for removal by subtractive IMAC
Enterokinase	DDDDK*	Secondary sites at other basic amino acids
Factor Xa	IDGR*	Secondary sites at Gly-Arg
Granzyme B	D*X, N*X, M*N, S*X	Nonspecific cleavage activity
Intein	Cleaves self	On column cleavage (chitin-beads)
PreScission	LEVLFQ*GP	Protease removed by Ni-chelating or GSH resin
Sortase A	LPET*G	Ca^{2+}-induction of cleavage, requires an additional affinity tag for on column removal
TEV protease	EQLYFQ*G	His-tag for removal of the protease
Thrombin	LVPR*GS	Secondary sites. Biotin labeled for removal

2.2 MATERIAL AND METHODS

2.2.1 Host and Vector Design

2.2.1.1 Expression Bacteria Strain and Reagents

The expression bacteria strain *E. coli* C43(DE3) was purchased from Lucigen (Middleton, WI). Strain C43(DE3) contains no intrinsic plasmids and expresses the T7 polymerase from the lacUV5 promoter upon IPTG induction. In addition, C43(DE3) shows no proteolytic activity towards exogenously overexpressed proteins (Miroux and Walker, 1996).

^{3}H-CP55,940 (specific activity: 88.3 Ci/mmol), CP55,940, WIN55,212-2 and SR144,528 were obtained from RTI International (Research Triangle Park, NC). Isopropyl-β-D-thiogalactoside (IPTG), Benzonase nuclease and lysozyme were purchased from EMD Chemicals (Gibbstown, NJ). Protease inhibitor cocktail was purchased from Sigma (St. Louis, MO). All restriction and DNA modifying enzymes were purchased from New England Biolabs (Ipswich, MA).

2.2.1.2 Construction of CB2 receptor expression vectors

The constructs used in the present study are shown in Figure 2.1. All expression vectors were based upon the pET-21a vector backbone. Gene fragment encoding octa histidine tagged Mistic (8His-Mistic) was derived from the pMIS3.0E vector via polymerase chain reaction using specific primers (For: 5'-ATATACATATGAAACACCACCACC-3'; Rev: 5'-

AAGCTTACCACTCAGGATCATGTAAT-3'). The forward and reverse primers included the restriction sites NdeI and HindIII respectively for subsequent cloning. The Human cannabinoid receptor 2 (CNR2) gene with the Factor Xa sequence (5'-ATTGAGGGACGC-3') fused at its 5' terminal end (Xa-CB2) was extracted from the pMMHb-TrpΔLE-Xa-CB2 vector using HindIII and BamHI sites. The pET-21a-TarCF construct was used as a template. The 8His-Mistic-Xa-CB2 encoding sequence was cloned upstream of the TarCF gene on the pET-21a-TarCF template using NdeI and BamHI sites. A Tobacco Etch Virus (TEV) (sequence 5'-GAAAACCTATACTTCCAAGGA-3') protease recognition site was introduced between TarCF and CB2 encoding sequences on the expression plasmid pET-21a for higher efficiency and specificity of protein cleavage. Similarly, the 8His-Mistic encoding sequence was subcloned into the pET-21a-CB2-TrxA template using NdeI and HindIII sites to create the construct (2). Constructs (3) and (4) were created by removing either the TarCF sequence (using HindIII and XhoI sites) or the 8His-Mistic sequence (using NdeI and AvaI sites) from construct (1), followed by subsequent Klenow treatments (or a subsequent Klenow treatment) and intramolecular ligation reaction. Double digestion of the constructs with AvaI and HindIII released the CB2 gene fragment confirming successful cloning. All construct sequences were verified by automated DNA sequencing at the University of Pittsburgh Genomics core facility.

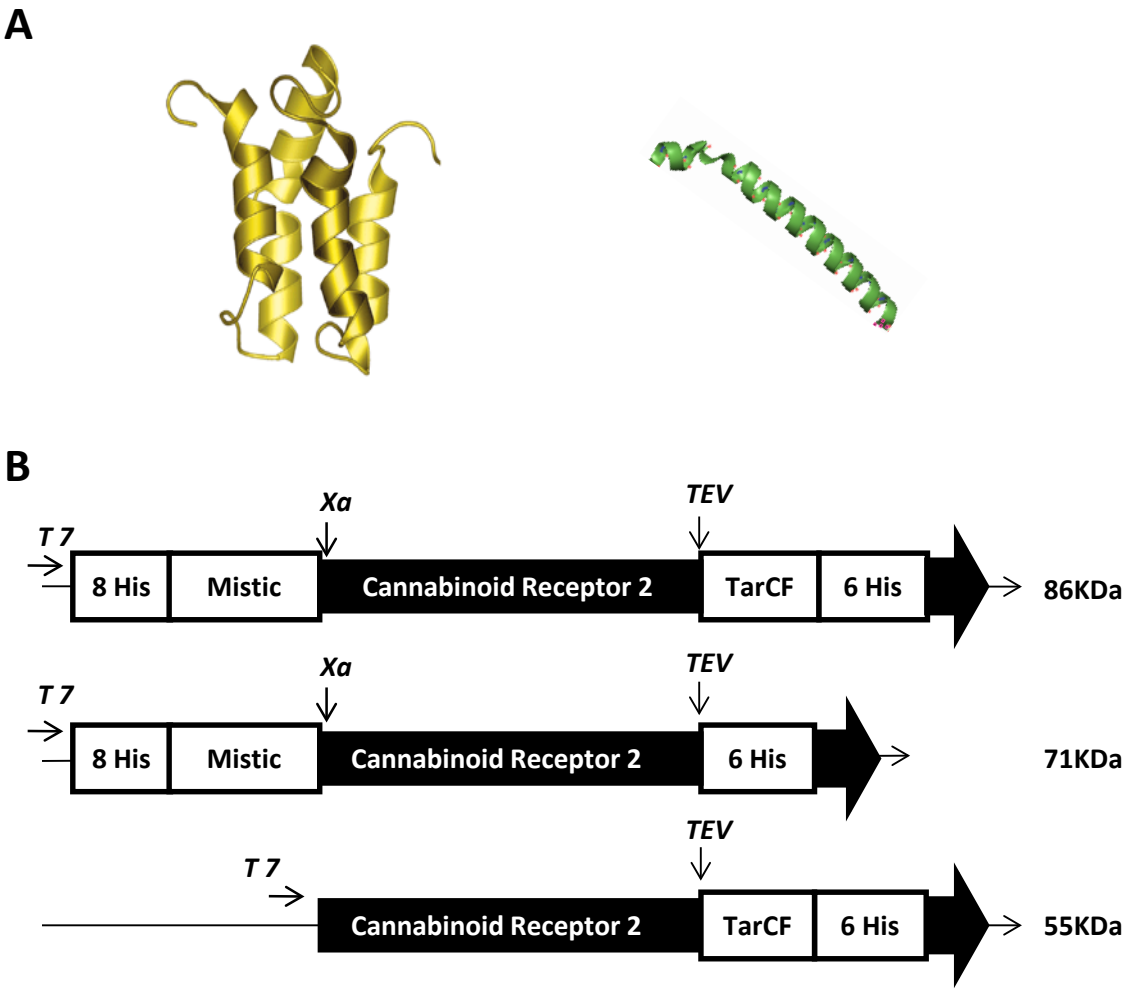


Figure 2.1. Fusion Partners and Expression Vectors for CB2 fusion protein production.

(A) Left: NMR structure of N′-terminal fusion partner Mistic (PDB:1YGM; golden). Right: Fragment (Ile139-Asn184) of crystal structure of TsrCF (PDB:2D4U) which is a homologue of the C′-terminal fusion tag TarCF, used in this study. **(B)** Schematic diagram of human CB2 fusion protein constructs. All expression plasmid vectors were constructed on the pET-21a vector backbone under the control of the T7 promoter. Mistic, the N-terminal fusion tag, was separated from CB2 by the Factor Xa sequence while TarCF, C-terminal fusion tag, were separated from the CB2 receptor by the TEV sequence. The boxes shown are not drawn to scale. TarCF, C-terminal fragment of bacterial aspartate chemosensory transducer Tar; TEV, tobacco etch virus sequence; His, Histidine residues.

2.2.2 Optimization of receptor production

2.2.2.1 Culture of *E. coli* C43(DE3) for protein expression

Minicultures were inoculated with single colonies from an LB-Ampicillin plate containing freshly transformed *E. coli* C43(DE3). The bacterial cultures were grown overnight in presence of Ampicillin (100µg/ml) in a shaker (at 250 rpm) at 37°C. Bacterial maxicultures (1 L) were inoculated with the minicultures and shaken at 250 rpm, 37°C until the culture reached an OD_{600} of 0.6. Expression of the recombinant CB2 protein was induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG), followed by continuous shaking for another 4 h at 37°C. Cells were harvested by centrifugation. After a 50 mM Tris–HCl (pH 8.0) wash, the pellets were stored at -80°C for further experiments.

Optimization of culture conditions and IPTG concentration were performed for maximum expression of Mistic-CB2-TarCF. Briefly, *E. coli* C43(DE3) cultures were grown to OD_{600} of 0.6, induced with 0.5 mM or 1 mM IPTG and then maintained at 25°C or 30°C for 8, 22, 32, 48 and 72 h after IPTG induction.

2.2.2.2 Preparation of bacterial membrane fractions

The harvested bacterial pellet was washed twice with 0.1 M Tris-HCI (pH 8.0) buffer and resuspended in the same buffer containing 20% (w/v) sucrose. The OD_{600} of the cell suspension was adjusted to 10.0. The suspended pellet was incubated at 37°C for 25 minutes in the presence of the Protease Inhibitor Cocktail (PIC) (430 µg/ml) and lysozyme (0.5 µl/g) followed by immediate addition of EDTA to a final concentration 10 mM. After a 0.1 M Tris-HCI wash

containing 20% sucrose, the pellet was then subjected to osmotic lysis by suspension in cold water and sonicated on ice. This suspension was incubated for 1 h with PIC, Benzonase nuclease and $MgCl_2$ (10 mM). After a low speed centrifugation (4500 × g, 10 mins), the supernatant was subjected to a high speed spin (100,000 × g, 90 mins) at 4°C. The membrane pellet obtained was dissolved in Tris-HCI buffer with 20% sucrose and PIC. This was flash frozen and the aliquots were stored at -80°C for subsequent use.

2.2.2.3 Detergent Screening

In an effort to determine the class of detergents that can effectively solubilize Mistic-CB2-TarCF from the extracted membrane fraction we used a set of 12 detergents (supplemented in buffers) to identify the detergent with the highest membrane protein solubilizing capability. We used DUAL extract membrane protein buffer set (DUAL systems Biotech, Zurich Switzerland) which composed of an even representation of 12 detergents of varied physicochemical properties. Membrane fractions from *E. coli* C43(DE3) expressing the Mis-CB2-TarCF were prepared as mentioned in 2.2.2.2. Equal amounts of Membrane fractions containing 100ug of total protein were aliquot to 12 high centrifugal speed resistant polycarbonate tubes. Membrane preparations were spun at 50000xg, 30 mins at 4°C. The supernatant containing the storage buffer was completely removed from pelleted membrane preparation. 200ul of detergent buffers (1-12) were added following complete solubilization of the pellet. Upon complete solubilization all tubes were incubated at RT in a slow orbital shaker. Samples were collected after 4hrs and overnight.

2.2.2.4 Detection of CB2 fusion protein expression in *E.coli*

Transformed *E. coli* cell pellets or membrane fractions were analyzed for CB2 expression by Coomassie brilliant blue (CBB) staining and Western blot. Cell pellets or membrane fractions were lysed in buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 10% SDS, 430 μg/ml PIC) and sonicated briefly. The lysate supernatants were subjected to SDS-PAGE, followed by Coomassie blue staining. For Western blot analysis, the lysate supernantant (30 μg) was heat-denatured, subjected to 12 % SDS-PAGE, and transferred to polyvinylidene fluoride membrane. Following, histidine tagged CB2 receptor were probed with anti-His monoclonal (1:1000, Sigma) and anti-CB2 polyclonal (1:1000, Cayman Chemicals) primary antibodies. The protein bands were detected using Amersham Enhanced Chemiluminescence-Western blotting detection reagents (GE Healthcare, Piscataway, NJ).

2.2.3 Determination of receptor pharmacological activity in *E. coli* membrane fractions

2.2.3.1 Saturation binding assay of the fusion protein

The saturation binding of 3H-CP55,940 to the membrane proteins was performed as described previously (Leifert et al., 2009). Briefly, the membrane fractions (20 μg) were incubated with increasing concentrations of 3H-CP55,940 (0.01–5 nM) in 96-well plates at 30°C with slow shaking for 1 h. The incubation buffer was composed of 50 mM Tris-HCl (pH 7.4), 5 mM

$MgCl_2$, 2.5 mM EGTA and 0.1% (w/v) fatty acid free BSA. Ligand was diluted in incubation buffer supplemented with 10% dimethyl sulfoxide and 0.4% methyl cellulose. Non-specific binding was determined in the presence of 1:1000 unlabeled CP55,940 (5000 nM) in excess. The reaction was terminated by rapid filtration through Unifilter GF/B filter plates using a Unifilter Cell Harvester (PerkinElmer). After the plate was allowed to dry overnight, 30 µl MicroScint-20 cocktail (PerkinElmer) was added to each well and the radioactivity was counted by using a Top Counter (PerkinElmer). Data from these assays were analysed using GraphPad Prism 5.0 Software. The difference between total and nonspecific binding equals the receptor specific binding. Non-linear regression analysis revealed the receptor density (Bmax) and the equilibrium dissociation constant (Kd) values of 3H-CP55, 940 for the CB2 receptor.

2.2.3.2 Competitive ligand displacement assay

CB2 receptor ligand displacement assay was performed as described previously (Leifert et al., 2009). The known CB2 ligands CP55, 940 (unlabelled), WIN55, 212-2 and SR144528 were used in this displacement assay to test whether the fusion proteins expressed in *E. coli* C43(DE3) exhibited receptor-ligand binding properties. Briefly, non-radioactive (or cold) ligands were diluted (10-2-103 nM) in binding buffer [50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2.5 mM EGTA and 0.1% (w/v) fatty acid free BSA], supplemented with 10% dimethyl sulfoxide and 0.4% methyl cellulose. Each assay plate well contained a total of 200 µl of reaction mixture comprised of 20 µg of membrane protein, labeled ^{3}H-CP55,940 ligand at a final concentration of 4 nM and the unlabeled ligand at its varying dilutions as stated above. Plates were incubated at 30°C for 1 h with gentle shaking. Reactions were terminated and read as described in the

previous section. All assays were performed in triplicate (n=3) and data points represented as mean±S.E.M. Bound radioactivity was analyzed for Ki values using non-linear regression analysis by GraphPad Prism 5.0 software.

2.3 RESULTS AND DISCUSSION

2.3.1 Expression of CB2 with fusion partners

Recombinant CB2 receptor produced in *E. coli* does not have the ability to translocate to the membrane and is devoid of membrane environment. This phenomenon has been proposed to be toxic towards the host and lead to misfolded protein aggregation, requiring the isolated protein to undergo refolding (Michalke et al., 2010). To enhance membrane protein expression and solubility with correct folding, as well as membrane localization of the recombinant GPCRs, researchers have employed several approaches including the identification of fusion partners linked with GPCRs in *E. coli* (Korepanova et al., 2005; Zuo et al., 2005). Previous studies have shown that MBP or thioredoxin (Trx) can stabilize and improve the expression and solubility of foreign fusion proteins in *E. coli* (Kapust and Waugh, 1999). Furthermore, Trx fusion proteins can be folded correctly and express complete biological activity (LaVallie et al., 1993). Mistic, a bacterial membrane-associating protein, has been found to enhance expression of eukaryotic

membrane proteins at the bacterial membrane (Blain et al., 2010; Kefala et al., 2007; Roosild et al., 2005). The chemosensory aspartate receptor, Tar, is a resident membrane protein of the bacterial host which is expected to facilitate membrane protein expression (Meir et al., 2010). Combining different fusion partners at both ends of a target gene has emerged as a promising strategy to facilitate expression and improve the solubility of recombinant proteins (Yeliseev et al., 2005). However, application of the fusion tags Mistic and TarCF for the expression of GPCRs in *E. coli* has not been investigated previously. For the first time, we report in this study the use of different fusion partner combinations (Mistic, TarCF and TrxA) for the functional expression of the recombinant CB2 receptor in *E. coli* C43(DE3). We show here, that the fusion protein Mistic-CB2-TarCF is overexpressed by *E. coli* and localized to the bacterial membrane with ligand binding properties comparable to those on mammalian cells.

2.3.2 Expression of Cannabinoid receptor 2 fusion protein in *E. coli*

E. coli C43(DE3) cells were transformed respectively with the fusion constructs shown in Figure 2.1. Expression of the recombinant fusion proteins were detected by Western blot or Commassie Blue staining. Since all constructs contain a multi-histidine tag, we used either anti-His or anti-CB2 antibody to detect expression of the CB2 fusion protein. As shown in Figure 2.2A, Mistic and TarCF alone failed to boost the CB2 gene expression. Only when both partners were linked to CB2 in the proper order did the fusion protein expression increase dramatically. In addition, fusion protein expression was also observed with the Mistic-CB2-TrxA construct at a comparable expression level with that of the Mistic-CB2-TarCF (Appendix B; Figure 4.15).

However, the construct Mistic-CB2-TarCF was used for further optimization due to its novel combination of fusion partners and prominent expression levels of recombinant fusion protein.

Next, we investigated whether the expressed CB2 fusion protein possessed membrane affinity or localized to the *E. coli* membrane. Coomassie staining of the membrane enriched fractions revealed a prominent band at MW ~ 86 kDa for the fusion protein Mistic-CB2-TarCF while no bands were detected for the fusion proteins that carried either the Mistic or TarCF tag individually (Fig. 2.2B). Importantly, the membrane enriched fractions exhibited the same expression pattern as the whole *E. coli* cell lysates indicating that all or the majority of CB2 fused protein driven by the two partners could localize or integrate into the *E. coli* membrane. Our data suggests that combination of the two tags (Mistic and TarCF) may contribute synergistic effects on the CB2 protein expression compared to either tag used alone. Our data also show that membrane fractions contain concentrated CB2 fusion protein compared to the whole cell lysate, indicating that most of the fusion protein is localized within the bacterial membrane. This is in accordance with previous studies where the effects of Mistic and other bacterial membrane resident protein to stabilize GPCR expression has been demonstrated (Baneyx, 1999; Freigassner et al., 2009; Kefala et al., 2007).

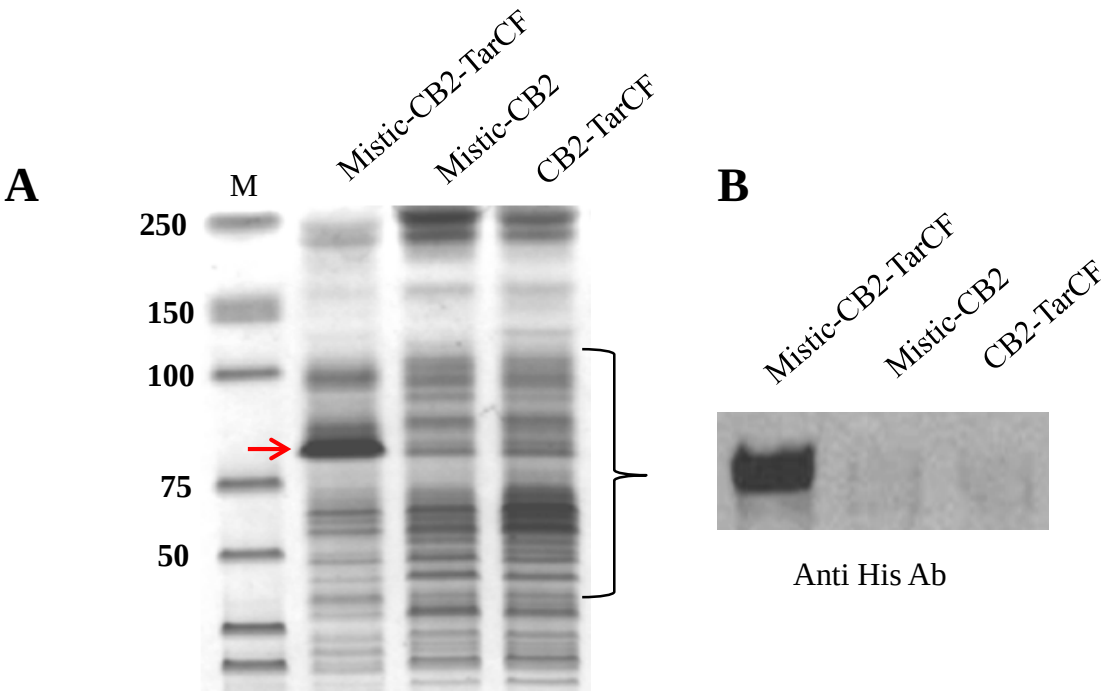


Figure 2.2. Expression of the CB2 receptor fusion protein

(A) Coomassie Brilliant Blue staining on the SDS–PAGE of extracted membrane fractions. The expected MW for the respective fusion proteins are as follows – Lane 1: Mistic– CB2–TarCF (86 kDa); Lane 2: Mistic–CB2 (71 kDa); Lane 3: CB2–TarCF (55 kDa). Red arrow show the corresponding Mistic– CB2–TarCF fusion protein expression. M: protein marker. Care was taken to normalize the amount of *E. coli* C43(DE3) membrane fraction sample loaded on the gel. **(B)** Representative immunoblot of His-tagged CB2 fusion protein detected in *E. coli* C43(DE3) membrane fractions using anti-His antibody. Membrane fractions loaded from left are Lane 1: Mistic–CB2–TarCF; Lane 2: Mistic–CB2; Lane 3: CB2–TarCF.

In absence of induction with IPTG, there was no expression of the fusion protein. However, after induction with IPTG, the fusion protein level increased significantly in a time-dependent manner (Fig. 2.3), suggesting that the expression cassette is under the tight control of the lac operon and T7/lac promoter and lacI gene in the pET21a. Control of recombinant protein expression under the tight regulation is necessary to avoid toxicity of protein expression to the host and ensure sufficient biomass of viable *E. coli* that would be available for membrane protein expression after induction.

To determine the time point of maximum receptor production, IPTG-induced cells were harvested at different time intervals from 1-8 hours. Whole cell lysates were analyzed by Western blot using mouse anti-His (1:1000 dilution) and rabbit anti-CB2 antibodies (1:500 dilution). As shown in Figure 2.3A, CB2 fusion protein expression levels steadily increased and reached maxima at 3-4 hours, followed by significantly reduced expression. The steady decrease in the expression level is due to the increased proteolytic cleavage of the produced protein. Thus, from this experiment we can conclude that the expression level of the fusion protein peaked during culture at 37°C for 3-4 hours after IPTG induction. Since IPTG induction at lower temperature was previously reported to improve the exogenous protein production and correct folding (Freigassner et al., 2009), we optimized the culture conditions by combining different IPTG concentrations (0.5 mM and 1 mM), culture temperature and time. We found that the expression levels of the fusion protein Mistic-CB2-TarCF are weakly detected during culture period (2 to 8 h) at 22°C (data not shown). The fusion protein expression at 30°C was not distinct from the regular 37°C culture condition (Fig. 2.3B). However, once the transformed cultured underwent IPTG induction (1 mM) at 25°C for 8 h, the fusion protein levels were significantly increased 2-fold of that of regular conditions (Fig. 2.3B). Overall, 0.5 mM IPTG used for

inducing protein expression resulted in lower amounts of fusion protein than 1 mM—especially for the conditions of culture temperatures at 25 or 30°C (data not shown).

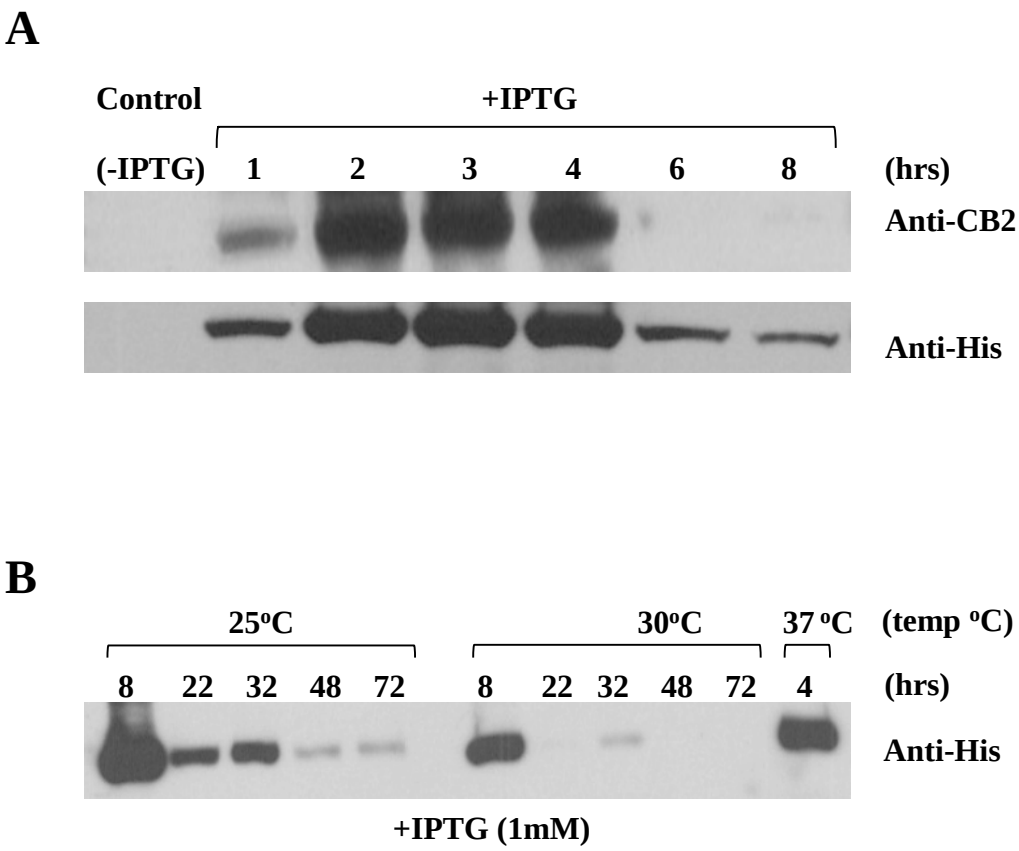


Figure 2.3 Optimization of conditions for fusion protein expression in *E. coli.* (A) Cells transformed with Mistic–CB2–TarCF were grown for the indicated hours after induction with IPTG (0.5 mM). Expression levels of fusion protein Mistic–CB2–TarCF tagged with poly-histidine were detected by Western blot with anti-CB2 or anti-His antibody. Control group (0 hrs) represents no IPTG induction. **(B)** Optimization of Mistic–CB2–TarCF fusion protein production in *E coli*. Different combinations of the parameters (IPTG, culture temperature and time) were tested and one representative is shown.

2.3.3 Receptor Saturation Binding Assay

pET-21a-Mistic-CB2-TarCF transformed *E. coli* membranes were subjected to a saturation binding assay to determine receptor saturation with increasing concentrations of ^{3}H-CP55,940. pET-21a-TarCF transformed *E. coli* membranes were used as the negative control. For the membrane proteins derived from Mistic-CB2-TarCF transformed *E. coli* , the maximal receptor density (B_{max}) and dissociation constant (Kd) of ^{3}H-CP55,940 for specific binding sites were 928.8 ± 117.6 fmol/mg protein and 3.04 ± 0.69 nM, respectively (Figure 2.4A). Membrane fractions clearly showed CB2 receptor binding characterization by the abundance of binding sites recognized by agonist ^{3}H-CP55,940. For the negative control, however, no difference was observed between specific and nonspecific binding (Figure 2.4B), indicating that the overwhelming majority of the total binding was contributed by the nonspecific binding. This confirms the absence of CB2 receptor on pET-21a-TarCF transformed *E. coli* membranes. A comparison of the specific saturation binding profile of Mistic-CB2-TarCF and the negative control CB2-TarCF is represented in the Appendix A Figure 4.9. The comparative plot highlights the difference of B_{max} values.

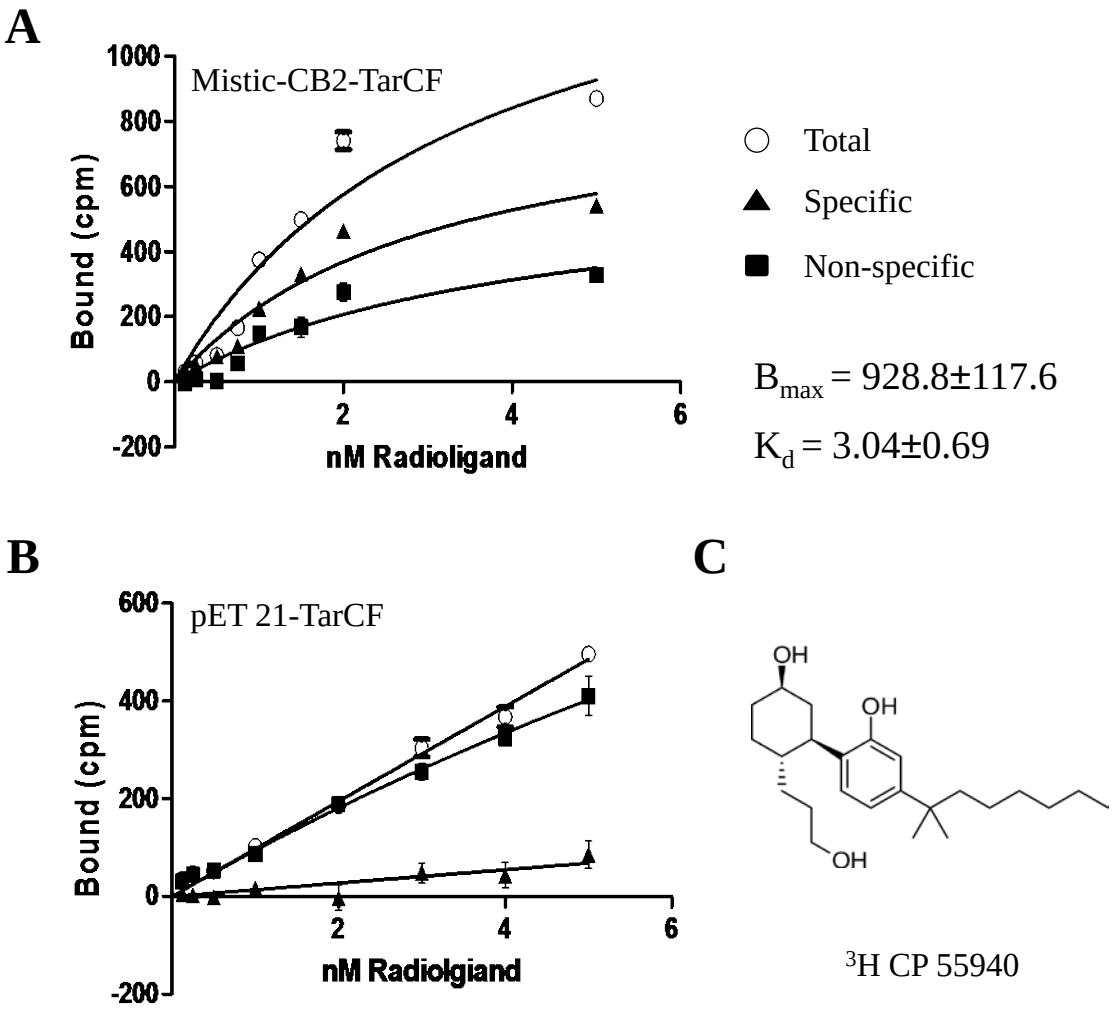


Figure 2.4 Saturation binding assay of the membrane fractions

Saturation binding assay was performed with membrane fractions by increasing the concentration of agonist, ^{3}H CP 55940 using a fixed amount of target protein. A 1:1000 excess of cold CP 55940 was added in the reaction mixture to account for non specific binding. Total (○) and non-specific (■) binding was measured and the deduced specific binding saturation isotherm (▲) was obtained as the difference between total and nonspecific binding. **(A)** Mistic–CB2–TarCF; **(B)** pET 21-TarCF (negative control). Assay was performed in triplicate (n = 3). Data presented as mean ± SEM. **(C)** Chemical structure of the non-classical Cannabinoid receptor agonist, ^{3}H CP 55940.

2.3.4 Receptor Competitive Binding Assay

The conformational state of a receptor protein determines the functional state of a receptor. High affinity binding between a ligand and its receptors is often physiologically important when a portion of the binding energy can be used to cause a conformational change in the receptor, resulting in altered downstream signaling pathways. In the present study, to confirm whether the expressed fusion proteins from the *E. coli* exhibit functional binding activity, we used well-known CB2 ligands to probe the interactions of these ligands with their cognate binding sites on the CB2 enriched membrane fractions (competitive binding assay), by quantifying the equilibrium dissociation constant (K_i). By using 10 µg of membrane fractions of Mistic-CB2-TarCF fusion protein in the binding assay, the Ki values for these ligands were well consistent with previous reports using the CB2 from mammalian cells: CP 55,940 (K_i = 1.43 nM), SR 144528 (K_i = 2.02 nM) and WIN 55212-2 (K_i = 0.13 nM). These results (Figure 2.5) indicate that the ligand binding domain of the CB2 receptor in the fusion protein is not perturbed by the physical presence of its neighboring fusion partners Mistic and TarCF.

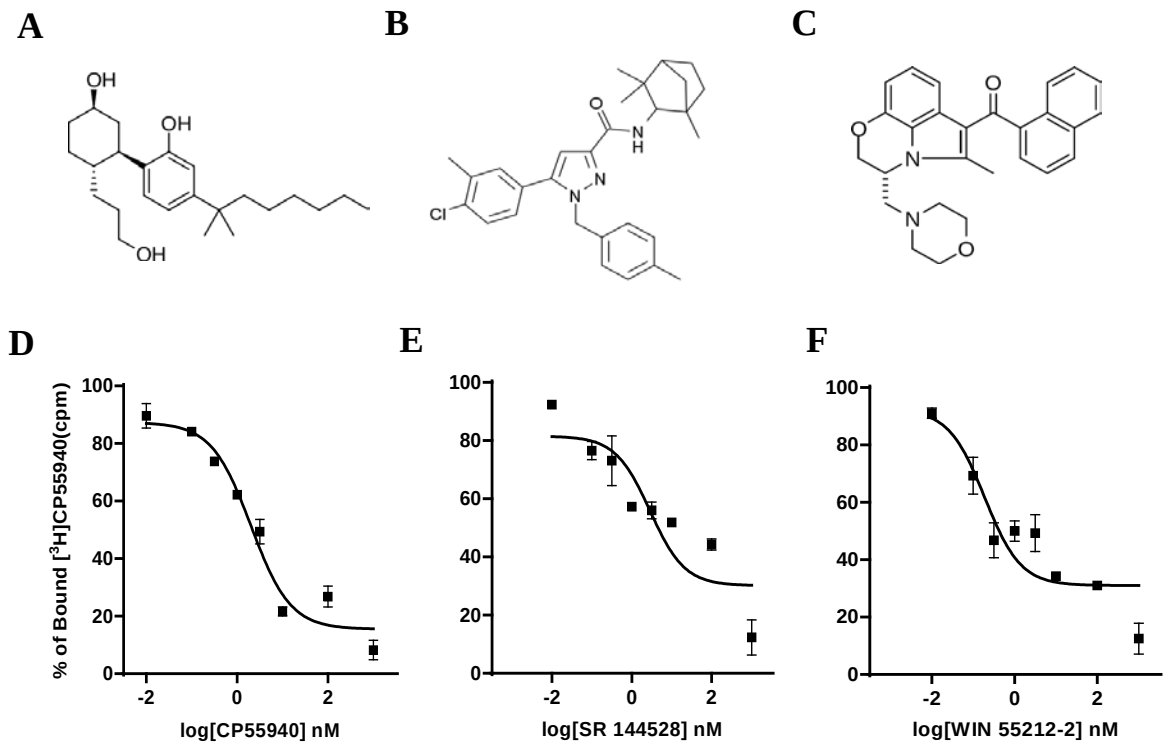


Figure 2.5 Competitive ligand displacement assay of the membrane fraction.

Upper Panel : Chemical structures of **(A)** non-classical Cannabinoid receptor agonist, ^{3}H CP 55940 **(B)** receptor inverse agonist SR144528 and **(C)** receptor agonist WIN55212-2. *Lower Panel:* Competitive displacement of the ^{3}H-CP55,940 was obtained by using an increased amount of cold ligands. Binding profile of **(D)** CP55,940 (unlabelled), K_i = 1.43 nM; **(E)** SR144,528, K_i = 2.02 nM; and **(F)** WIN55,212–2, K_i = 0.13 nM. Assay was performed in triplicate (n = 3). Data represented as mean ± SEM.

2.3.5 Detergent Extraction of Mis-CB2-TarCF

The capability of a particular detergent to extract fusion protein from the membrane depends on the combination of several physicochemical properties. These include the overall hydrophobicity of the Membrane protein (or its fusion construct), length of the fatty acyl chain and the size of the polar head group. In an effort to compare the extraction efficiencies of different classes of detergents, we used Ionic, Non-Ionic, Zwitter Ionic and Alkylsaccharide detergents to extract Mis-CB2-TarCF from membrane fractions. As shown in Figure 2.6, alkylsaccharides and zwitter ionic class of detergents showed a higher efficiency of extraction. Results clearly indicate the presence of a much heavier band for the Mis-CB2-TarCF at ~85kDa for these detergent classes with slightly better extraction efficiency for the alkylsaccharides. Interestingly these detergents also show increased extraction efficiency for the contaminant protein observed at ~60kDa. This observation is consistent with previous attempts for detergent extractions of membrane bound GPCRs (Yeliseev et al., 2007; Yeliseev et al., 2005).

We understand that the targeting of fusion partners to the bacterial membrane is critical to the conformational stability of the expressed CB2 protein. The possible role of the fusion partners for the overexpression and stabilization the CB2 protein is illustrated schematically (Figure 2.7) for easy comprehension. Two different dispositions of the TarCF can possibly lead to the stabilization of the fusion construct – one with the alpha helical segment of the TarCF extending into the cytosol (Figure 2.7A) and the other to the transmembrane region (Figure 2.7B). In this putative model, the CB2 receptor structure was adapted from the 3D CB2 model reported previously by Xie et al. [5], while the structure of Mistic and Tsr (structurally related to Tar) were determined by NMR (PDB:1YGM) [40] and cryo-electron microscopy [55] studies,

respectively. However, confirming the putative model will be subject to further biophysical studies.

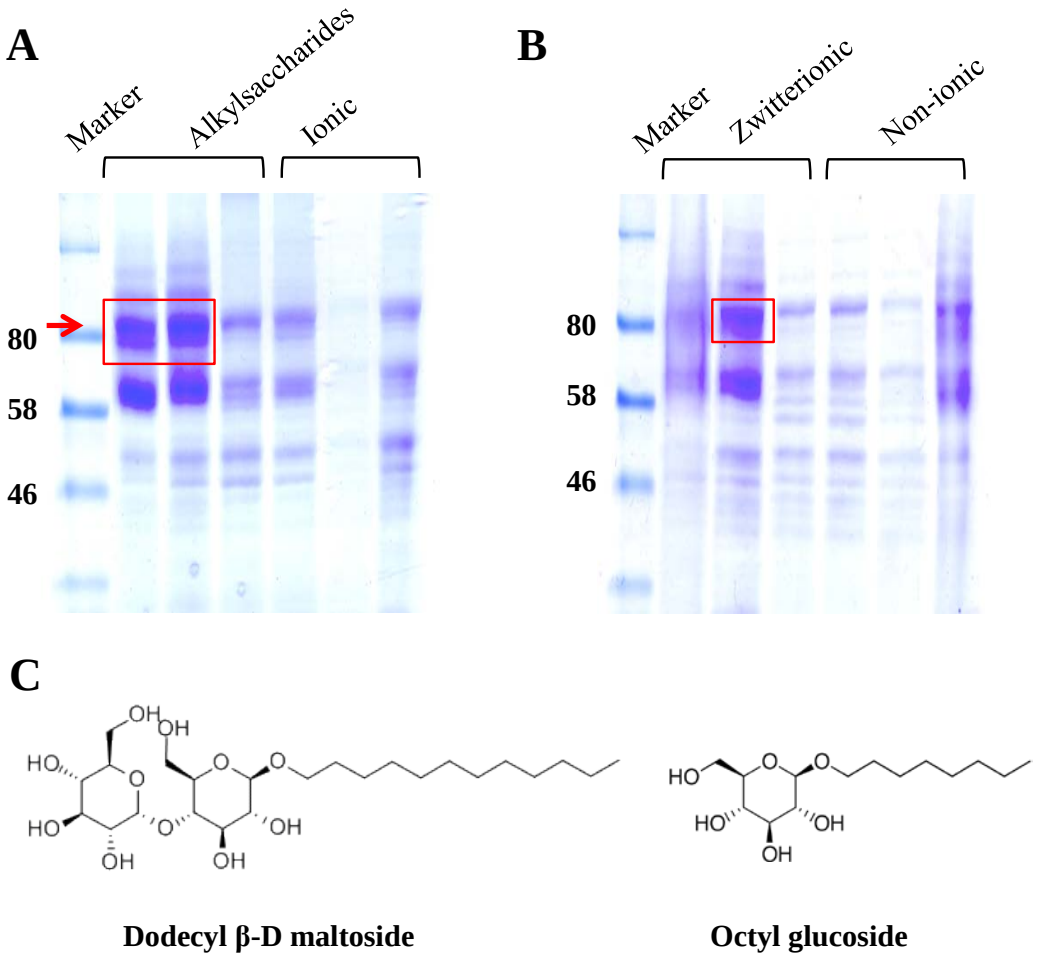


Figure 2.6. Detergent screening for extraction of Mis-CB2-TarCF

Screening for solubilization was done using a representative set of 12 detergents which vary widely in their physicochemical properties - Ionic, Non-ionic, Zwitterioninc or alkylsaccharaides by nature (DUAL extract membrane protein buffer kit, Dual systems Biotech). **(A)** and **(B)** Coomassie Briliant Blue staining of SDS-PAGE of solubilized membrane fractions of *E. coli* C43(DE3) transformed with Mistic-CB2-TarCF. **(C)** Chemical structure of alkylsaccharides that solubilized the extracted membrane most effectively (*highlighted in red on PAGE*).

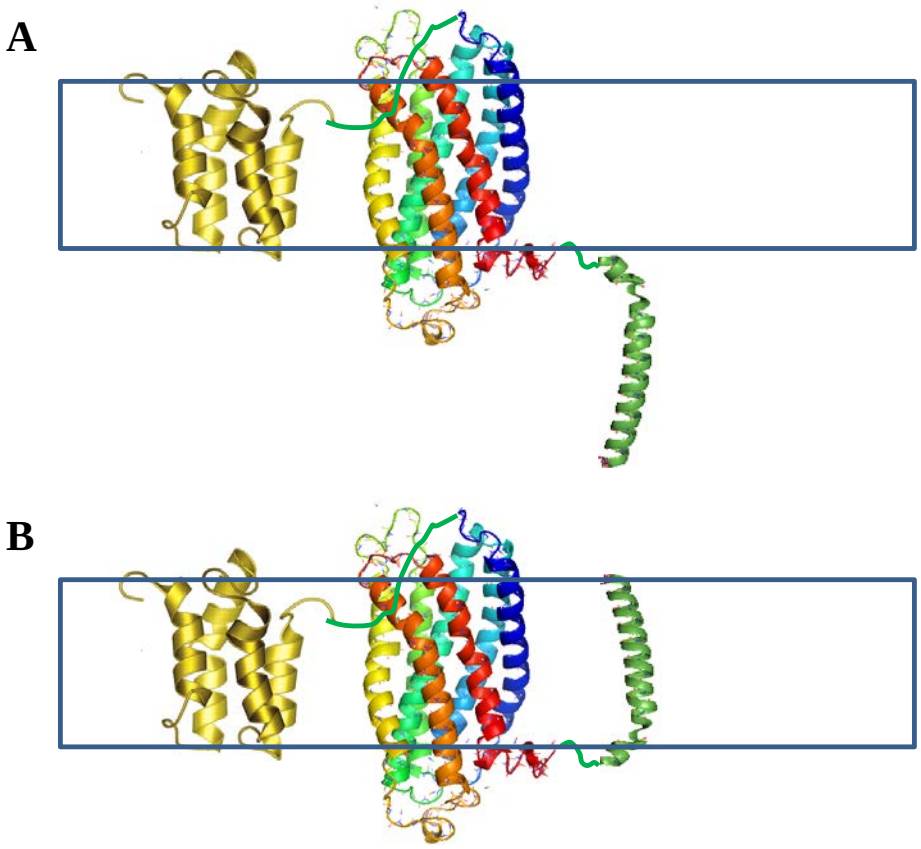


Figure 2.7. Putative models of the Mistic-CB2-TarCF within the *E.coli* inner membrane

Model showing two possible modes of orientation of the TarCF in the Mis-CB2-TarCF,which may lead to a stabilized structure of the fusion protein **(A)** Mistic-CB2-TarCF located within the lipidic bacterial inner membrane with the C′-terminally fused TarCF tag extending into the cytosol. **(B)** TarCF fragment can fold onto itself and extend into the lipid bilayer of the *E. coli* inner membrane. Hydrogen bonding interactions stabilizing the alpha helix would be more stable in the lipid environment (low dielectric constant).

2.4 CONCLUSION

The Mistic-CB2-TarCF expression construct can produce functional CB2 receptor in *E. coli* C43 (DE3) membrane. We observed several novel attributes of the fusion partners Mistic and TarCF. Mistic and TarCF can together boost the expression of CB2 to a much higher level compared to Mistic or TarCF alone. Using the tag partners in combination it was observed that majority of the fusion protein translocated to the bacterial inner membrane. Further we observed a saturable binding pattern of the CB2 ligand, ^{3}H – CP55940 which is a clear indication of the receptor mediated binding event. Competitive Ligand Binding was observed with different subclasses of CB2 ligands. All these data taken together indicate that functional CB2 receptor can be produced in the bacterial membrane. Despite the functionality of the membrane bound CB2, the expression level of the protein in bacteria is very low. This is in accordance with our observations that SDS PAGE CBB staining of the whole cell lysates from bacteria reveal little or no expression of the protein, whereas when enriched membrane fractions were used, moderate expressions levels were noted by SDS-PAGE. This posed as a huge roadblock towards moving in the direction of extraction and purification of the expressed protein. In our small scale detergent screening, we observed that Mistic-CB2-TarCF bands can be extracted with alkylsaccharide class of detergents. However we used enriched membrane fractions as starting material. Very less starting material was obtained at the start of the chromatographic processes. In our pilot scale chromatographic steps we observed very low elution of the specific protein; which is probably due to the presence of small amounts of the protein of interest in the starting load material and also the low capture efficiency of the protein in the presence of detergents. With these reasons combined we determined that using an expression system with much higher yields of CB2 would allow moving forward for purification and characterization steps. Hence

we moved to the approach of inclusion body directed expression of the CB2, which will be discussed in the following chapter.

3.0 EXPRESSION AND PURIFICATION OF THE CB2 FROM INCLUSION BODIES

3.1 INTRODUCTION

3.1.1 Inclusion Bodies

Inclusion bodies (IBs) are protein aggregates, about 0.5-1.3μm in diameter, formed in the cytoplasm or sometimes in the periplasm due to over expression of a protein in bacteria. Combination of several factors result in the formation of these highly dense (~1.3mg/ml) aggregates that are predominantly amorphous in character. The threshold concentration of the protein that is believed to lead to inclusion body formation is ~2% of the total cellular protein. Proteins having disulfide bonds are usually prone to form inclusion bodies since the formation of disulfide bonds is inhibited by the reducing cytoplasmic environment. Also the formation of inclusion bodies are more pronounced when the protein of interest is hydrophobic. The concentration of a protein of interest within the inclusion bodies is very high. Inclusion bodies can be easily isolated following cell disruption. In composition the inclusion bodies generally have a highly hydrated formation.

3.1.2 Advantages and disadvantages of GPCR expression as inclusion bodies (IBs)

The expression of GPCRs as inclusion bodies in the *E. coli* results from the incapability of the bacterium to handle and process the expressed recombinant protein. Formation of inclusion bodies in a cell is undesirable, yet there are some advantages associated with the formation and localization as IBs in *E. coli.* The formation of IBs can be determined as the presence of refractive particles in the cytoplasm (Margreiter et al., 2008). One of the main advantages of producing GPCRs, or for that matter, any heterologous gene product as IBs is that they are mostly enriched with the recombinant protein (99%). However the IBs may also contain chaperones and membrane fragments. The IBs self-associate to form tight clusters, thereby masking the proteolytic sites on the proteins. Hence IBs are generally refractory to proteolytic cleavage. They are usually mechanically very stable and thus can be isolated and enriched from the cell preparations by centrifugation. For receptors like mouse Cannabinoid Receptor 1(muCB1), human Parathyroid Hormone Receptor 1(huPTHR1)(Michalke et al., 2010), Chemokine receptor (Park et al., 2006) the yield of membrane protein receptors would increase manifolds if expressed as inclusion bodies. The applicability and the success of the method to produce and generate “native like” GPCR depends on the combined success and method development of firstly the IB directed expression, purification and secondly the development and availability of methods to refold the obtained IBs. These challenges will be discussed in the following sections.

3.1.3 Current methods for Inclusion Body directed expression

Once a protein is expressed in large quantities as insoluble inclusion bodies, it can then be solubilized and refolded to gain functionality. The method to produce relatively soluble protein from these insoluble aggregates has had a significant commercial consequence when it was used to produce therapeutic proteins and peptides (Marston, 1986). The composition of efficient *in-vitro* refolding environment are available in the REFOLD database (www.refold.med.monash.edu.au) (Chow et al., 2006). Refolding strategies are generally based on the use of strong denaturing detergents to extract the protein from the IBs and then successively exchange them with milder detergents to allow them to refold (Misawa and Kumagai, 1999). The necessity of the fusion partner for directing the expression of some proteins as IBs is counterintuitive. Expressed protein in the bacteria exist in the equilibrium of properly folded, partially folded and aggregates or inclusion bodies and are all subjected to the actions of chaperons and proteases (Villaverde and Carrio, 2003). Fractions which exist in the partially folded state are much more susceptible to proteolytic degradation hence a fusion partner attachment is provided to minimize protein yield loss in cases where the protein will not be expressed in the soluble fraction. (Hwang et al., 2013). Ideal fusion partners for the generation of IBs typically include proteins with an overall hydrophobicity and the tendency to form beta sheets (Fernandez-Escamilla et al., 2004). Some of the most common fusion partners are the Ketosteroid isomerase (KSI), EDDIE, PagP, TrpΔLE and GST.

The KSI fusion partner is a 14 kDa protein (Kamerlin et al., 2010) that is available in the pET13b expression vector from Novagen. The protein is extremely hydrophobic and has a strong tendency to accumulate with the fusion partner as inclusion bodies. The KSI fusion system has been used well for the expression of the antimicrobial peptides which are rendered inactive in

their IB state. Antimicrobial peptides have also been expressed using several other fusion partners e.g. the truncated *E. coli* PurF fragment (Lee et al., 2000) and the histone fold fragment of the human transcription factor TAF12 (Vidovic et al., 2009). The N^{PRO} derived from the N-terminal auto protease, is derived from the classical swine fever virus (CSFV) and is also known as EDDIE (Achmuller et al., 2007). The N^{PRO} tagged protein is expressed both as inclusion bodies and as soluble proteins within the *E. coli.* Insoluble, tagged fusion was dissolved in Guanidine HCl and was then dialyzed in refolding media to remove the GuHCl. The N^{PRO} is cleaved off and removed while in the refolding process. This is an example where following inclusion body directed expression the tag can be removed enzymatically without any requirement for chemical cleavage.

Another recently developed method to have IB directed expression of recombinant proteins involve using the PagP expression system (Hwang et al., 2012). PagP is a bacterial membrane protein which localizes itself in the outer membrane of Gram negative bacteria. It is structurally similar to many bacterial outer membrane proteins in having a beta-barrel with the hydrophobic amino acids facing the lipids and the hydrophilic residues making up the hydrophilic inner core. Surprisingly, in spite of the high content of beta-barrel structure, PagP is not very hydrophobic making it easier for solubilization. Mutations that excise the membrane localization signal from this protein makes it an ideal choice for the expression of this protein as a fusion partner for IB directed expression (Booth and Curran, 1999).

The TrpΔLE expression system is an example of a fusion partner developed early on and leads strongly towards IB directed expression of the fusion partner (Landick et al., 1985). The bacterial Trp operon consists of a leader sequence- TrpL composed of a 14 amino acid leader sequence (MKAIFVLKGWWRTS) followed by a stop codon. A large deletion was made from

that encompassed a portion of the TrpL leader sequence and the start of the first protein coding sequence TrpE. This resulted in the formation of the fused polypeptide known as TrpΔLE (TrpΔLE). The TrpΔLE is composed of 17 amino acid residues and includes the N-terminal end of the TrpΔLEader sequence fused in frame with the carboxy terminal fragment of TrpE. A remarkable increase in the amount of protein expression was noticeable when the TrpΔLE was fused to insulin, somtostatin and TGF-α (Derynck et al., 1984). The application of the TrpΔLE as a fusion tag has become significantly widespread and has been used for the production of several small peptides and also some small membrane proteins (Cook et al., 2011). The impetus to use the TrpΔLE as a fusion partner in our study was corroborated by the recent development of methods to refold GPCRs from IBs (Michalke et al., 2010).

The Glutathione S Transferase is a 26 kDa protein derived from the *Schistosoma japonicum* and has been frequently used for a one step purification of many different fusion proteins (Smith and Johnson, 1988). The purification is conducted by the binding of the GST to immobilized glutathione which can then be released by the addition of 10mM of reduced glutathione. The advantages of having the GST fusion partner are more than just purification. The GST tag allows for enzymatic detection of the protein purified protein and may protect the protein from the proteolytic digestion by sequestering the GST tagged proteins as dimers/oligomers. The GST tag may allow for the soluble expression of proteins however GST tag is considered to be a poor "solubility enhancer" as tagging with the GST partner may lead to a combination of soluble, insoluble or partially soluble proteins. GST tagged protein purification is a matter of consideration for the extraction and purification of inclusion bodies particularly when denaturing conditions are employed. Denaturing conditions would result in conformational disruption of the GST and it will not be able to bind the immobilized glutathione. However mild

denaturing conditions can be used with the GST tag. This has been a cause of concern which has been exploited in the expression and purification of the CB2 receptor, in the present study. The GST fusion tag is bulky and is generally needs to be removed for proper folding, activity and crystallization of the fused partner protein. Most expression vectors like pGEX expression vector (GE Healthcare) contains the gene encoding a protease cleavage site e.g. Thrombin, Factor Xa or TEV (PreScission) proteases. The GST tagged protein after cleavage can be removed from the reaction mixture by subtractive IMAC using a charged GSTrap column.

3.1.4 Current methods for solubilization and purification of Inclusion Bodies

Solubilization of the hydrophobic IB by denaturant or detergents and the removal of the expression/ fusion partners by proteases are the essential steps towards the purification and refolding steps. The procedure and the use of a few different denaturing agents will be discussed in this section. Although we have implemented milder detergents in our study, this section will have a brief overview of all the possible denaturant/ detergent solubilization methods available. The discussions about the particular solubilizing method implemented for the CB2 in our present study (using SDS and Sarkosyl) is mentioned methodologies section 3.2. In general, due to the hydrophobic nature of inclusion bodies, solubilization is the key step for the isolation of the inclusion bodies. The solubilizing detergent may be used in the subsequent steps of purification and protease cleavage. Guanidine Hydrochloride at 6 Molar concentration is often a good choice as a denaturant since it does not lead to chemical modifications on the amino acid residues in the protein. It can also be used during IMAC purification. However it is not compatible with SDS-PAGE. On the other hand, urea has the advantages of being cheaper and compatible with SDS-

PAGE. Urea has its own disadvantages since it can form isocyanate ions on long term storage which can carbamylate the amino groups in a protein.

Following solubilization, the protein of interest is available in the liquid phase for purification. In general the first step involves purifying the solubilized protein by exploiting the affinity of the fused tag to its ligand that is immobilized on a purification column. This step concentrates the protein of interest and isolates it to high degree of homogeneity upto as much as 90-95%. Commonly used expression tags include polyhistidine, GST, MBP. The Immobilized Metal Affinity Chromatography (IMAC) – which includes Ni^{2+} is one of the best choices at this step for several reasons. Firstly the polyhistidine tag is usually made up of only six Histidine residues and generally does not interfere with the protein's structure and /or activity when left fused (not removed from the protein by chemical/ enzymatic cleavage) even after purification or renaturation. Secondly, the tag solely works on the interaction of the charged divalent cation of nickel and localized electron pair on His residue (Ni^{2+}-- Histidine interaction). Hence it is compatible with most of the denaturing conditions like Urea, Guanidine Hydrochloride and upto 1% SDS. Other commonly used tags are the Glutathine-S-transferase (GST) or the Maltose Binding protein (MBP) that rely on the secondary structure of the protein tag and also have greater conformation dependent binding compared to 6X Histidine. However these tags can be used in a milder detergent containing buffer.

3.2 MATERIAL AND METHODS

3.2.1 Expression and purification of CB2 with the TrpΔLE partner

3.2.1.1 Expression bacteria strain and Reagents

The expression bacteria strain *E. coli* C43(DE3) competent cells was purchased from New England Biolabs (Ipswitch, MA). The base vector, pMMHb plasmid was a kind gift from Dr. Stanley Opella at the University of California San Diego. Isopropyl-β-D-thiogalactoside (IPTG), benzonase nuclease and lysozyme were purchased from EMD Chemicals (Gibbstown, NJ). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Sigma Aldrich (St. Louis, MO). Enzyme Factor Xa was obtained from New England Biolabs (Ipswitch, MA). AKTA machines and chromatographic media were obtained from GE Healthcare. Detergents Triton X-100 was purchased from Pierce, Dodecyl- β-D-maltoside (DDM) and octyl glucoside were purchased from Affymetrix. All general chemicals (like SDS, Tris-HCl and salts) were obtained from Fisher Scientific (Pittsburgh, PA). Antibodies to His tag and CB2 were purchased from Santa Cruz Biotechnologies and Thermo Fisher respectively.

3.2.1.2 Expression Vector design and construction

The expression vector for the TrpΔLE fusion expression of the CB2, pMMHb-TrpΔLE -9His–Xa-CB2 was previously constructed in our laboratory. Construction of the expression vector and expression of CB2 receptor transmembrane fragments are described in several publications (Xie et al., 2004; Zheng et al., 2006; Zheng et al., 2005). The construct (Figure 3.1A) was designed to separate the CB2 from the TrpΔLE leader by the Factor Xa enzymatic cleavage site. Verification of the expression vector was conducted by double digestion of the parent gene with HindIII and

BamHI to release the CB2 gene fragment. The released product was gel purified and sequence verified as CB2 receptor cDNA. All sequences were verified by automated DNA sequencing at the University of Pittsburgh Genomics core facility.

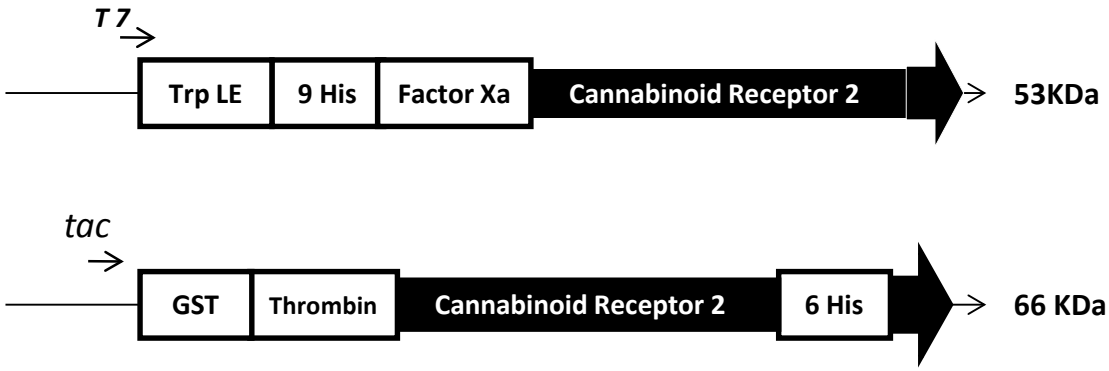


Figure 3.1. Expression vectors for inclusion body directed expression of the CB2 receptor

Schematic diagram of human CB2 fusion protein constructs. Expression plasmid vectors were constructed on the pET-21a vector backbone under the control of the T7 or *tac* promoter. The CB2 receptor was fused to either TrpΔLE or a GST tag and was separated by Factor Xa or Thrombin cleavage sites respectively. Both the constructs carried either a N′-terminal (9) His tag or a C′-terminal (6) His tag. The boxes shown are not drawn to scale. TrpΔLE - TrpΔLE fusion leader**,** ; GST, Glutathione-S-Transferase; His, Histidine residues.

3.2.1.3 Expression and purification of the TrpΔLE-9His-Xa-CB2

Small scale expression of TrpΔLE-9His-Xa-CB2

Minicultures (5ml) were inoculated with single colonies from an LB-Ampicillin plate containing *E. coli* C43(DE3) freshly transformed with the expression construct pMMHb-TrpΔLE-9His-Xa-CB2. Midicultures (25ml) were inoculated with the minicultures and grown until the culture reached an OD_{600} of 0.7. All bacterial cultures were inoculated in a 1:100 ratio (v/v) of the (saturated culture) : (fresh media) in presence of Ampicillin (100 µg/ml) and grown in a shaker (at 250 rpm) at 37^0C. Expression of the recombinant CB2 protein was induced with 1 mM isopropyl-β -D-thiogalactoside (IPTG), followed by continuous shaking for about another 3 hrs at 37^oC. Cells were harvested by centrifugation.

Preparation, enrichment and solubilization of Inclusion bodies

Cell pellets were resuspended in 100 ml lysis buffer (0.1 M Tris–HCl, pH 7.5; 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) supplemented with Lysozyme and Benzonase nuclease). The slurry was tip sonicated for 5 mins under low or medium output control (3-8, Fischer Scientific Dismembrator Sonifier) with the sample on ice and followed by centrifugation at 48,000xg for 20 mins at 4°C. Supernatant was discarded. Pellets from the spin were washed twice in 0.1 M Tris–HCl, pH 7.5; 10 mM EDTA and 1 M NaCl. The slurry was resuspended in 40 ml of extraction buffer (0.1 M Tris–HCl, pH8.0; 10 mM sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), and 1 mM EDTA overnight at room temperature. Solubilized proteins were separated from the insoluble material by centrifugation at 48,000g for 20 min at room temperature and dialyzed against 0.1 M sodium

phosphate (pH 8.0) and 10 mM SDS. The solubilized enriched IB preparation was tested for the presence of the TrpΔLE-His9-Xa-CB2 fusion protein by Western Blotting. Briefly, the load material was separated on an 8% SDS-PAGE gel, transferred to a PVDF membrane and the blot was probed with Anti His and Anti CB2 antibodies.

Large scale preparative pH gradient purification of TrpΔLE-9His-Xa-CB2

For a larger scale purification method development, cultures from about 4 liters were pelleted and were processed as described in the previous section. The solubilized enriched inclusion body preparation was subjected to extensive dialysis against loading buffer (0.1 M sodium phosphate, 10mM SDS, pH 8.0). At least two changes of the dialysis buffer were done followed by a high speed spin and filtration of the loading material. Load material was loaded onto a 10-ml Ni^{2+} affinity column (His Trap GE Healthcare) pre-equilibrated with loading buffer. After five successive column volume (CV) washes with the loading buffer at pH 8.0 and pH 7.0, the receptor was eluted in the same buffer at pH 6.0. The eluent fractions were analyzed by SDS-PAGE Coomassie Brilliant Blue Staining. Fractions containing our protein of interest were pooled together for further purification. A cation exchange chromatography on a SP FF column (GE Healthcare) was used for this. The column was equilibrated with 0.1M Tris-HCl, 10mM SDS, 1M NaCl to generate a charged matrix. The collected material was loaded onto the pre-equilibrated and charged SP FF column. The eluent from the ion exchange chromatography was concentrated upto a maximum of 10mg/ml in a volume of ~5ml and was loaded into a Hi Load Superdex column pre-equilibrated with 20mM Tris-HCl at pH 8.0. This buffer condition is suitable for the enzymatic cleavage of the Factor Xa enzyme. Peak corresponding to the protein of interest was collected and dialyzed against cleavage buffer (20mM Tris-HCl, pH 8.0).

Factor Xa Cleavage

The manufacturer recommendations state that 1µg of Factor Xa enzyme can cleave 50µg of the recombinant protein at 20mM Tris-HCl (pH-8.0) supplemented with 100mM NaCl and 2mM $CaCl_2$. Referring to the recommended concentration of Factor Xa as 1U (enzyme unit), reactions were set up over a range of concentrations (0U, 0.02U, 0.5U and 1.0U) of Factor Xa enzyme. Reactions were incubated by mixing the recombinant protein with the Factor Xa. Aliquots (equal volume containing 50µg of the recombinant protein – corrected for the dilution caused by Factor Xa) were collected at regular intervals of 3, 6, 9 and 16 (overnight) hours. Negative controls were maintained by collecting the recombinant protein diluted in the cleavage buffer without factor Xa at all the time points to account for any self cleavage.

3.2.2 Expression and purification of CB2 with the GST partner

3.2.2.1 Expression Bacteria strain and reagents

The expression bacteria strain *E. coli* BL21 competent cells was purchased from New England Biolabs (Ipswitch, MA). The base vector, pGEX 2T-M plasmid was obtained from Addgene (Addgene plasmid # 1128). All PCR and DNA modifying enzymes were purchased from New England Biolabs (Ipswitch, MA). Isopropyl-β-D-thiogalactoside (IPTG), benzonase nuclease and lysozyme were purchased from EMD Chemicals (Gibbstown, NJ). Protease inhibitor cocktail was purchased from Sigma (St. Louis, MO). Enzyme Thrombin was obtained from GE Healthcare. Detergents N-Lauroyl sarcosinate (Sarkosyl) was purchased from Amresco (Solon, OH), Dodecyl- β-D-maltoside (DDM) from Affymetrix and SDS from Fisher Scientific. 1,2-

Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) lipid was purchased from Avanti Polar Lipids. Glass filter subtype B (GF/B) was obtained from Perkin Elmer. AKTA machines and chromatographic media were obtained from GE Healthcare. ^{3}H-CP55,940 (specific activity: 88.3 Ci/mmol), CP55,940, WIN55,212-2 and SR144,528 were obtained from RTI International (Research Triangle Park, NC). All other chemicals, unless otherwise mentioned, were obtained from Fisher Scientific (Pittsburgh, PA).

3.2.2.2 Vector Construction of the pGEX 2T- GST-Thrombin-CB2-His 6

The expression vector used in this study was based on the pGEX2T-M vector backbone that houses the GST tag sequence followed by a thrombin cleavage site. The parent vector was double digested by BamHI and EcoRI restriction enzymes for subsequent cloning. The construct used in the present study is shown in Figure 3.1B. The human cannabinoid receptor 2 (CNR2) gene was PCR amplified from the pcDNA3.1 3HA-CB2. The forward and reverse primers (Forward 5'- AAG CTT GGA TCC ATG GAG GAA TGC TGG GTG ACA G -3' Reverse 5'- AAG CTT GAA TTC CTA TTA ATG GTG ATG GTG ATG GTG ATC AGA GAG GTC TAG ATC TCT G-3') included the restriction sites BamHI and EcoRI respectively for subsequent cloning. The reverse primer was designed to remove the last Cysteine residue from the CB2 gene and include the 6 Histidine (6His) tag sequence upstream of the stop codon and the restriction site. The amplified CB2 sequence was double digested with the corresponding restriction enzymes and ligated into the doubly digested pGEX vector in frame downstream of the GST-Thrombin sequence. The final construct contained the CB2 receptor with a N' terminal GST tag (separated by Thrombin cleavage site) and a C' terminal 6-Histidine tag. All construct sequences

were verified by automated DNA sequencing at the University of Pittsburgh Genomics core facility.

3.2.2.3 Expression and Purification of the GST –Thrombin-CB2-6His

Small scale expression of GST –Thrombin-CB2-6His

Minicultures (5ml) were inoculated with single colonies from an LB-Ampicillin plate containing *E. coli* BL21 (or BL21 codon plus) freshly transformed with the expression construct pGEX2T-GST-Thrombin-CB2-6His. Midicultures (50ml) were inoculated with the minicultures and grown overnight. Maxicultures (1 L) were inoculated with the midicultures and grown until the culture reached an OD_{600} of 0.8. All bacterial cultures were inoculated in a 1:100 ratio (v/v) of the (saturated culture):(fresh media) in presence of Ampicillin (100 µg/ml) and grown in a shaker (at 250 rpm) at 37^0C. Expression of the recombinant CB2 protein was induced with 0.8 - 1 mM isopropyl-β -D-thiogalactoside (IPTG), followed by continuous shaking overnight for about another 16 hrs at 27^oC . Cells were harvested by centrifugation and the pellets were stored at -80^0C for further experiments. The protocol for expression and purification of the GST CB2 was adapted from elsewhere (Park et al., 2012; Park et al., 2006).

Preparation, enrichment and solubilization of Inclusion bodies

Cell pellets were lysed by using a combination of mechanical and chemical lysis. Pellets were thawed on ice and lysed in buffer (20 mM Tris-HCl, 500 mM NaCl, 15% glycerol, pH 8.0) in a ratio of 50 ml lysis buffer per liter of cell pellet. Pellets were resuspended to homogeneity by

using a glass rod or by pipetting up and down gently. The slurry was tip sonicated for 5 mins under low or medium output control (3-8, Fischer Scientific Dismembrator Sonifier) with the sample on ice. 1mg Lysozyme (Fisher Scientific) was added to the sonicated mixture followed by 30 minutes incubation at room temperature with continual stirring. The slurry was centrifuged at 20, 000 rpm for 20 mins at 4°C. The pellet material was further resuspended in 20mM Tris-HCl supplemented with 0.5% Triton X-100 followed by homogenization to remove any large particulate debris. The homogenized material was centrifuged again as the previous step and the supernatant discarded. This process was repeated upto three times to finally obtain the pellet material enriched with GST-CB2 inclusion bodies (IB).

IB pellets were solubilized into the solution phase in a binding buffer (20 mM Tris-HCl, 500 mM NaCl, 1% Sarkosyl, 10 mM immidazole, 10mM β-mercaptoethanol, pH 8.0). All the pellet materials were completely solubilized into the solution phase, gently and were then homogenized in ice to breakdown larger debris followed by vigorous stirring for 1 hour at room temperature. Addition of additional binding/ solubilization buffer can be done at this stage if the material is too viscous. Lower volumes of solubilization buffer will not be effective to extract all the IB material. On the contrary, large excess of the solubilization buffer will also lead to the dilution of the load material. The solubilized material contained all the IB and the insoluble cell debris and DNA. This was centrifuged at high speed 25000 rpm for 1 hr at 4°C. The supernatant collected from this step contained the solubilized IBs and other hydrophobic proteins. The supernatant material was collected and treated with DNA breaking enzymes (Pierce Universal Nuclease) in the ratio of 10U/ liter of culture. This incubation step was carried out at 4°C for half of an hour. Following, the load materials was filtered through a 0.45 micron filter.

Large scale preparative purification of GST-Thrombin-CB2-6His

Purification of the solubilized IBs was carried out under denaturing conditions using a Ni^{2+} column (His Trap FF/ HisTrap FF crude GE Healthcare). The column was pre-equilibrated with the binding buffer and the flow through was collected while loading. The column was washed with 10 column volume of the binding buffer to completely remove any unbound material from the column. The column was then washed with binding buffer supplemented with 50mM Immidazole to washout the non-specifically bound proteins and enrich the column with the GST-CB2. Next, 1000Units of the Thrombin Protease was injected to the column and incubated overnight at RT. The next morning the cleaved CB2 protein was eluted from the column in the cleavage buffer supplemented with 500mM Immidazole. A charged GST Trap was attached beneath the HisTrap column during the elution from the His trap. The GST Trap column to be used was washed with both 6M Guanidine Hydrochloride and 70% Ethanol to remove any nonspecifically bound proteins and/ hydrophobic contaminants followed by complete wash by 1X Phosphate buffer saline (PBS). The attachment of the GST column with the HisTrap while elution was intended to to trap the uncleaved GST-CB2 and the free cleaved GST.

Following elution from the IMAC column the eluted fractions were tested for the presence of the cleaved (and the percentage cleaved) CB2. The fraction with the cleaved CB2 were pooled together and concentrated using an AMICON 30 kDa cut off concentrator (Millipore). The concentrated protein was further purified by Size Exclusion Chromatography (SEC) Superdex FPLC 26/60 (GE Healthcare). The column was pre-equilibrated with SEC buffer (20 mM HEPES, 0.5% SDS, 50 mM NaCl, pH 7.3). The fractions from the SEC was collected and were analysed by SDS PAGE and CBB staining if the samples get too dilute due to dilution from the SEC column the samples may be concentrated by TCA DOC precipitation to

get observable data from the SDS PAGE CBB staining. Similar to a SDS PAGE gel samples collected were also separated in a native PAGE gel.

Thrombin Cleavage

Thrombin cleavage reaction of the GST-Thrombin-CB2-His6 was carried out on the affinity column prior to specific elution. However several stages of optimization were implemented and excess of the protease was used for the cleavage reaction. Following the elution of the non-specifically bound proteins, the buffer was exchanged in the column with the Thrombin protease Cleavage buffer (20mM Hepes, 250mM NaCl, 0.1% Dodecyl-beta-D-maltoside, pH-8.0). To ensure complete buffer exchange and removal of any remaining denaturants (Sarkosyl and β-mercaptoethanol) from the column, it was washed with 20 CV of the cleavage buffer. Next, 1000 Units of the restriction grade thrombin protease (GE Healthcare) was resuspended into 1 CV of the thrombin cleavage buffer and injected into the column. The column was closed at both ends and incubated overnight at RT. Following incubation with the protease, the column was reconnected to the AKTA system, line equilibrated and A_{280} absorbance was corrected to baseline level. After about 10CV washing with the cleavage buffer, the bound cleaved CB2 was eluted from the column with cleavage buffer supplemented with 500mM Immidazole. Fractions were individually analyzed on 10% SDS-PAGE. Densitometric analysis was done to determine the fraction of uncleaved and cleaved GST CB2 protein.

Figure 3.2 shows a schematic flow diagram of the steps for the overall purification as designed and planned for the production of functional CB2 protein.

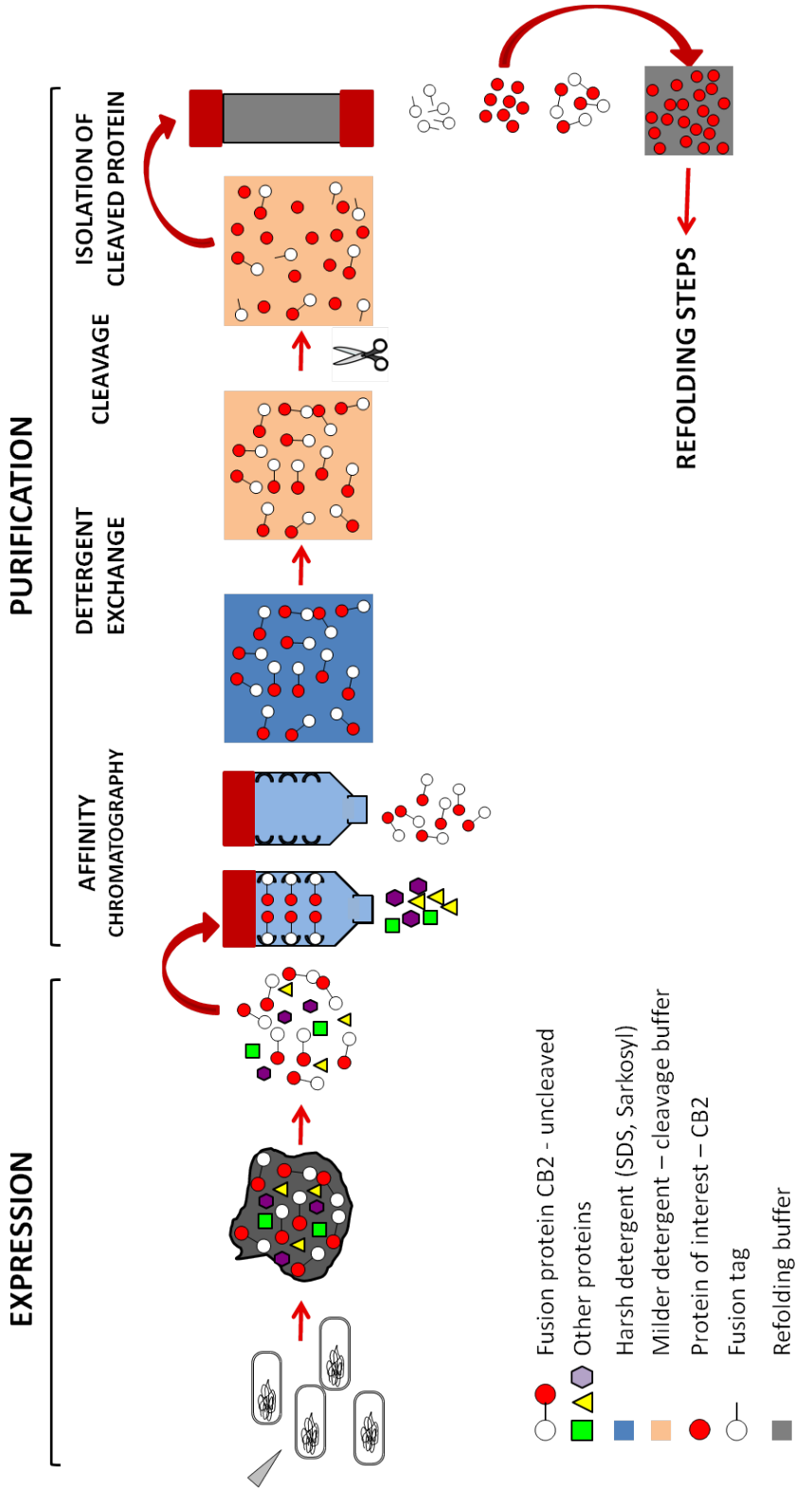


Figure 3.2. Overall steps involved in the extraction and purification of CB2 receptor inclusion bodies.

Overall steps involved in the extraction and purification of CB2 receptor inclusion bodies. Extracted IB pellets from transformed bacteria would be first captured in the affinity column and processed for detergent exchange and protease cleavage. The cleaved protein of interest can be isolated by size exclusion chromatography. The process is depicted left-right.

3.3 RESULTS AND DISCUSSION

3.3.1 Expression and Purification of CB2 in fusion with TrpΔLE tag

E. coli C43(DE3) cells were transformed with the fusion construct shown in Figure 3.1A. The recombinant protein was isolated from the bacteria as inclusion bodies and extracted therefrom using 10mM SDS. Figure 3.3 shows a schematic flow diagram of purification and cleavage steps towards the generation of cleaved CB2 receptor in batch on a small scale experiment. In the first step of purification, a simple pH gradient IMAC chromatography was conducted (Appendix A Figure 4.10). TrpΔLE-9His-Xa-CB2 was eluted from the IMAC column at pH 6.0, however it also contained other contaminating proteins at ~30 and 37 kDa as determined by SDS PAGE CBB staining (Figure 3.3A). To further purify and clean up the protein from IMAC, TrpΔLE protein was subjected to size exclusion chromatography using a Superdex column with little improvement of the protein purity (Figure 3.3B).

The TrpΔLE-Xa-CB2 demonstrates a very high expression level of the fusion protein. The fusion protein can be purified in a relatively simple pH gradient chromatography and further purified and polished through the ion exchange and the SEC. However, we observed that the protein could not be purified as effectively in the immidazole gradient chromatography. The reason for this behavior by this construct is not well understood. Combined ion exchange and Size exclusion chromatography instead, allowed for the generation of a pure fusion protein which was used for the subsequent cleavage reactions.

To determine the buffer conditions which may be more suitable for Factor Xa cleavage, the recombinant TrpΔLE-Xa-CB2 was exchanged using a 5ml ZebaSpin exchange system (Pierce) from 10mM SDS to different buffer environments. Cleavage efficiency was tested a 10X

concentration of Factor Xa in 10mM SDS, 0.8% Triton X 100, 8mM Octyl Glucoside and 8mM DDM. As shown in Figure 3.3C, no appreciable loss of fusion protein was noticed in the exchanged detergent environments (Figure 3.3C).

Further, the purified fusion protein was subjected to cleavage reactions in Triton X-100 in absence or presence of 10U of Factor Xa for 15, 45, 60, 90 and 120 minutes. Maximal cleavage of the fusion protein was observed between 60-90 minutes (Figure 3.3D). The cleavage mixture was incubated on a small scale with Nickel beads which leads to the removal of uncleaved proteins and isolation of the cleaved CB2 protein (Figure 3.3E).

Developed purification process was implemented for the large scale expression and purification of the TrpΔLE-CB2 fusion proteins with the inclusion of an additional ion exchange chromatographic purification step (Figure 3.4). Initial pH gradient IMAC led to the isolation of the TrpΔLE –CB2 fusion protein with contaminating proteins (Figure 3.4A). The TrpΔLE- CB2 protein did not bind to charged ion exchange column and was collected in the flowthrough fraction. However this allowed us to capture and remove several contaminants on the column except only the presence of one at 30 kDa (Figure 3.4B). Following this step, the nearly pure TrpΔLE-CB2 fusion protein was subjected to size exclusion chromatography using a Superdex HiLoad column (Figure 3.4C).

Factor Xa cleavage reaction of the TrpΔLE-9His-Xa-CB2 was challenging due to the hydrophobic nature of the protein and the susceptibility of the Factor Xa towards detergents. Factor Xa is derived from mammalian origin which works in a relatively aqueous environment. The structure of the protease enzyme and hence the activity is by obvious reasons, hampered in a hydrophobic milieu. On the other hand, the buffer containing high amounts of detergent is required to keep the fusion protein in solution.

To this end, it was necessary to carry out two imperative optimizations of the cleavage reaction. Firstly, the optimal amount of Factor Xa enzyme to be used for cleavage was required to be determined. Secondly, it was important to determine the optimal buffer conditions (detergent to be supplemented to carry out the cleavage reaction). All conditions were determined in batch to carry out the reactions with the control of time, temperature and buffer conditions and perform the cleavage reactions with the minimum amount of protein. In an effort to determine the ratio of μg of protein per units of Factor Xa, the cleavage reaction was first designed to try out different amount of factor Xa protease ranging from 0 to 1 enzyme units. Factor Xa treatment cleaves the 53 kDa fusion protein, TrpΔLE-9His-Xa-CB2 into two fragments TrpΔLE-9His (13kDa) and the CB2 receptor (40kDa). The Factor Xa enzyme has a molecular weight of 43 kDa. According to the manufacturer's protocol, 1U of enzyme per 50μg of the protein is recommended as optimal. Using 0U, 0.02U, 0.5U and 1.0U concentrations of the protease, successful cleavage and the appearance of cleaved bands were noticed only at 1U concentration (Appendix A Figure 4.11). Increasing the amount of enzyme may compensate for its decreased activity in the hydrophobic buffer and also account for any steric occlusion that may result from the buried nature of the cleavage site in a relatively less denaturing environment.

This cleavage efficiency and the high amounts of both the detergent and Factor Xa made it more difficult to carry on with this fusion construct. The principal reason of failure and complications can be attributed to the high degree of hydrophobicity that is conferred from the TrpΔLE fusion partner. We faced a catch 22 situation where the solubilization of the fusion construct required high amounts of detergents which is not favored by the protease and its activity is lost or reduced dramatically. The TrpΔLE system was hence not pursued further towards the cleavage, detergent exchange and refolding.

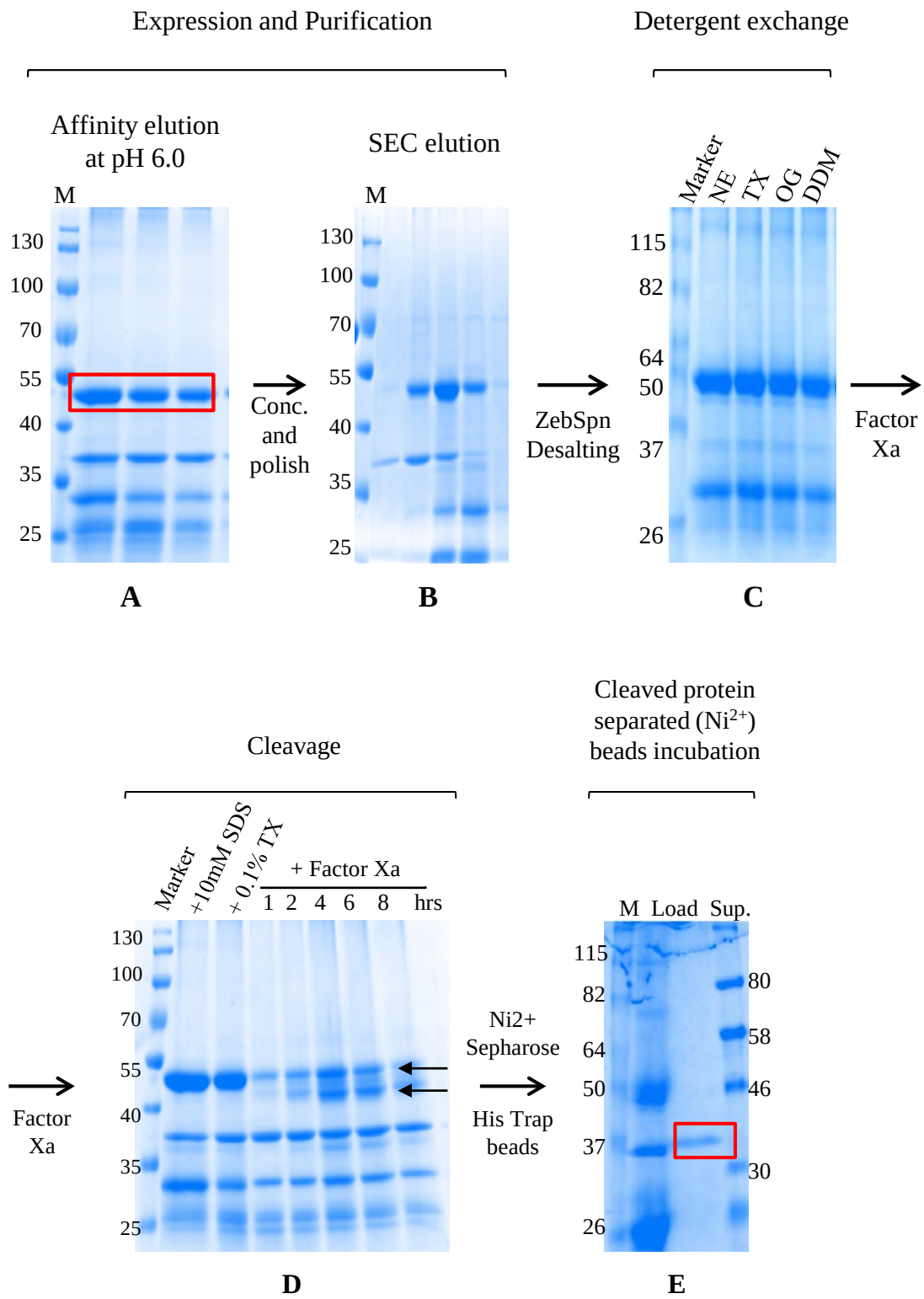


Figure 3.3. Expression, purification, detergent exchange and cleavage optimization of the TrpΔLE -CB2 fusion protein in batch

Expression, purification, detergent exchange and cleavage optimization of the TrpΔLE - CB2 fusion protein in batch. TrpΔLE leader fragment was used to direct the overexpression of CB2 into inclusion bodies. **(A)** CB2 receptor inclusion bodies were purified by pH gradient affinity chromatography (Ni^{2+}) column. The protein of interest (TrpΔLE -9His-CB2 : 53KDa) was polished by size exclusion chromatography (SEC) **(B)** following detergent exchange **(C)** with Triton X 100 (TX), Octyl Glucoside (OG), Dodecyl maltopyranoside (DDM) or no exchange (NE) to determine the amount of protein retained in different detergent solutions. **(D)** The fusion protein in TX was treated with Factor Xa protease for different incubation times and **(E)** cleaved product separated by subtractive IMAC using Ni^{2+} sepharose beads.

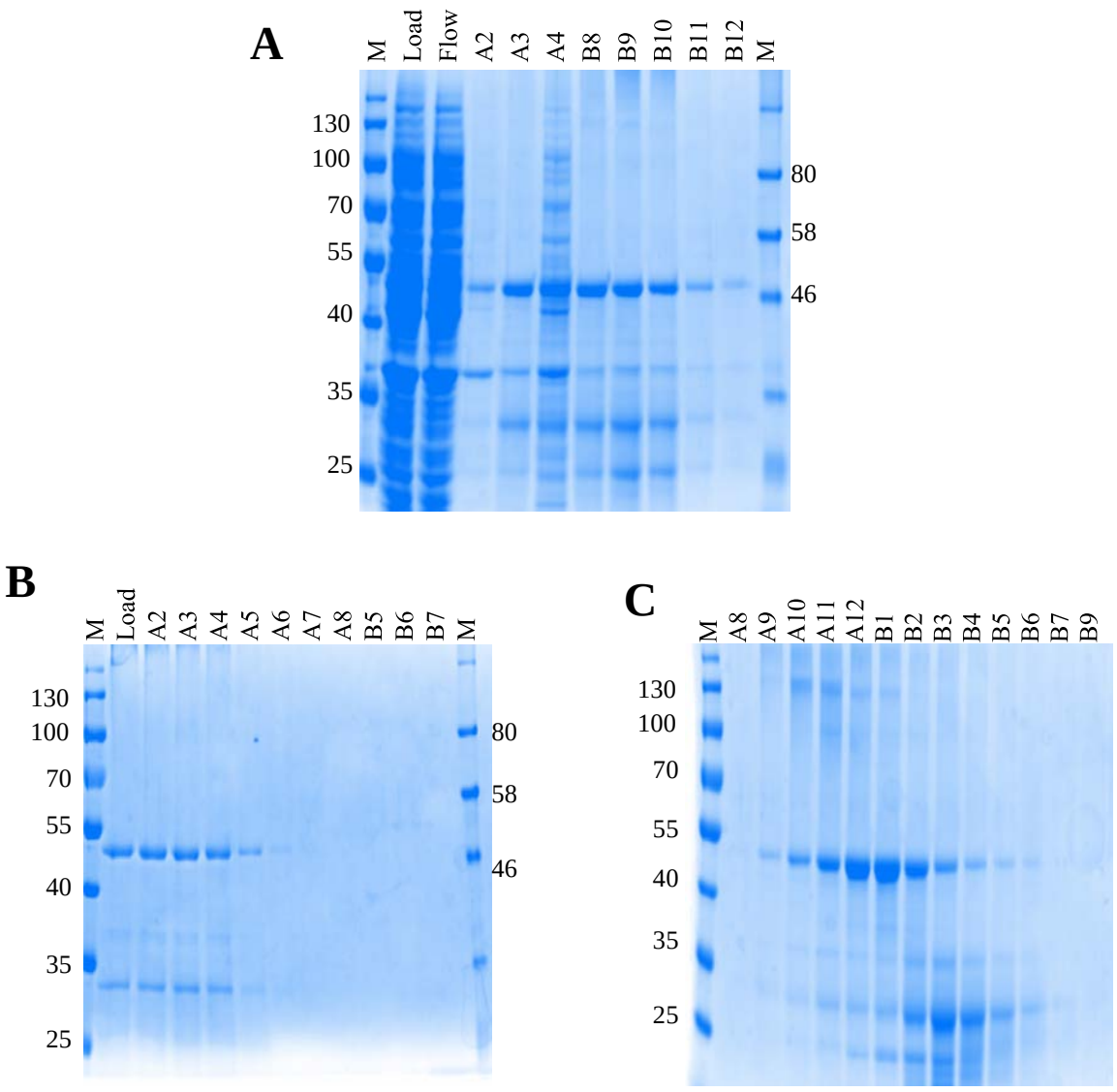
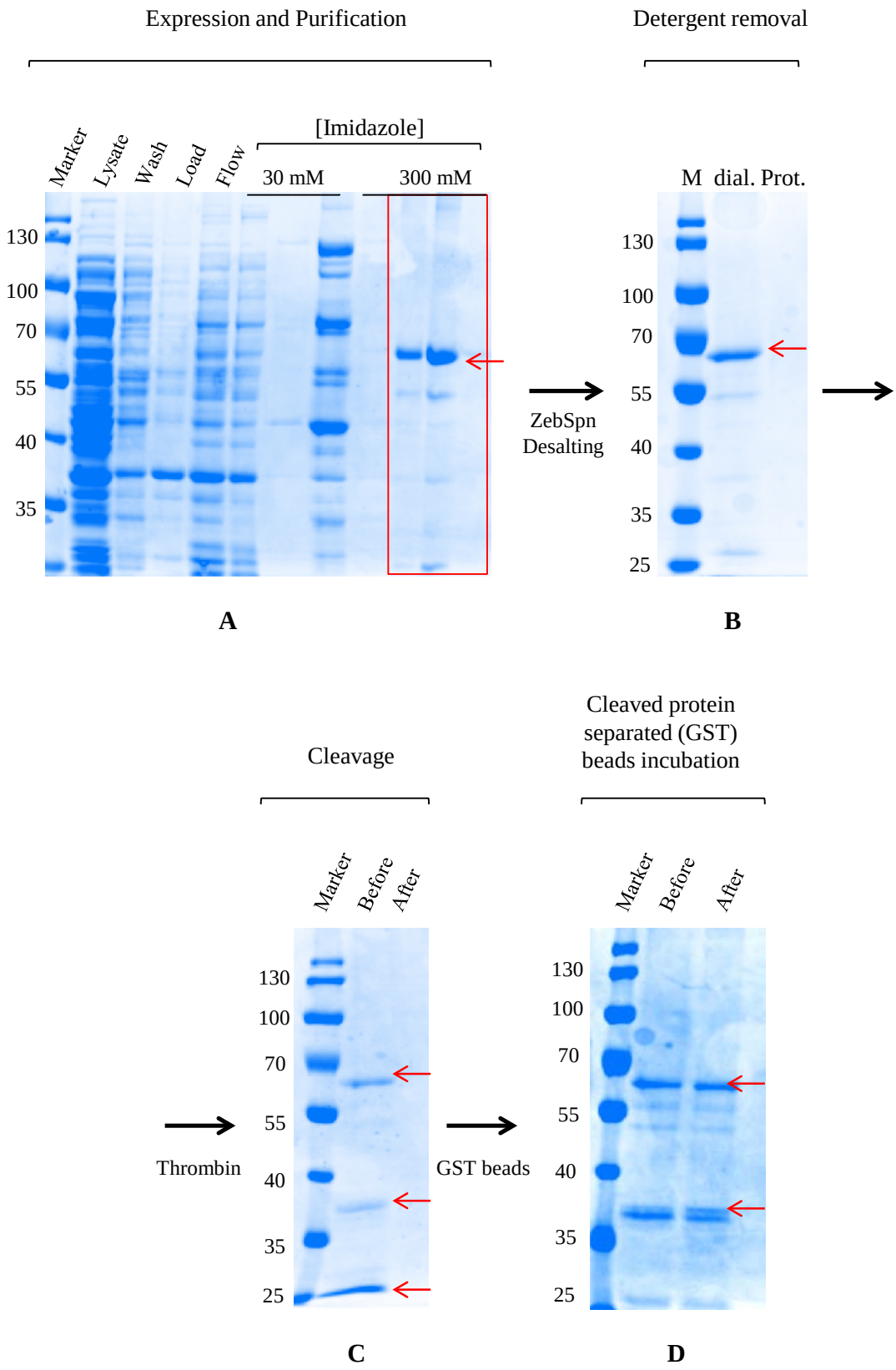


Figure 3.4. Scaled–up purification steps for the SDS solubilized TrpΔLE -CB2 fusion construct

The fusion protein solubilized in SDS was purified stepwise by **(A)** pH gradient IMAC chromatography. Proteins were loaded to HisTrap at pH 8.0 and the POI eluted at pH 6.0. **(B)** Subtractive ion exchange chromatography using an SP-FF column. POI collected in the flowthrough. **(C)** Preparative Size Exclusion chromatography. Protein obtained post size column was ≥ 80-90% pure.

3.3.2 Expression and purification of CB2 in fusion with GST tag

E. coli BL21 cells were transformed with the fusion construct shown in Figure 3.1B. The recombinant protein was isolated from the bacteria as inclusion bodies and extracted therefrom under denaturing conditions in presence of 1% Sarkosyl. Figure 3.5 shows results from the steps involved in a small scale (in batch) purification, detergent exchange and cleavage procedures of CB2 receptor from the inclusion bodies enriched with the fusion GST-Thrombin-CB2- 6His protein (66 kDa). In the first step of purification as shown in figure 3.5A, the solubilized enriched inclusion bodies of CB2 fusion protein was purified by IMAC using a Ni^{2+} column. Elution with 300 mM Imidazole yielded the protein to a good degree of purity. As shown in figure 3.5B, detergent was exchanged from 1% Sarkosyl to 0.1% DDM by using the ZebaSpin buffer exchange column and no significant loss of the GST CB2 was observed. Partial cleavage of $\geq$ 60% was achieved using the Thrombin protease under these conditions (Figure 3.5C). Separation of the cleaved and uncleaved protein was attempted by incubating the cleavage reaction mixture with GST beads. The reaction mixture was centrifuged and the supernatant subjected to SDS-PAGE and Western Blotting. CBB staining revealed the presence of successfully cleaved CB2 in the supernatant (Appendix A Figure 4.12; *Panel A*). Immunoblot analysis with anti-His (Figure 3.5D), anti-CB2 and anti-GST (Appendix A Figure 4.12; *Panel B*) antibodies confirmed the presence of successfully cleaved CB2 in the supernatant. Thus, GST beads were effective in the removal of the uncleaved GST-CB2 and the free released GST protein from the cleaved population in the reaction mixture.


Expression and Purification
Detergent removal
[Imidazole]
Marker
Lysate
Wash
Load
Flow
30 mM
300 mM
130
100
70
55
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ZebSpn
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130
100
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35
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A
B
Cleavage
Cleaved protein
separated (GST)
beads incubation
Marker
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Thrombin
GST beads
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D

Figure 3.5. Expression, purification, detergent exchange and cleavage optimization of the GST-CB2 fusion protein in batch

(A) CB2 Receptor Inclusion (GST-CB2) bodies were extracted by detergent Lauryl Sarkosyl and were purified by affinity chromatography (Ni^{2+}) column. **(B)** The purified protein of interest (GST-CB2-His6 : 66KDa) was exchanged using a Zeba Spin to remove the detergent Lauryl Sarkosyl with 0.1% DDM. **(C)** Thrombin cleavage was carried out for 16 hour at RT and incomplete cleavage was noticed. This yielded a mixture of parent protein, cleaved product and the released GST tag. **(D)** This mixture was incubated with GST-Sepharose beads for 1 hour at room temperature under mild agitation to separate the cleaved and uncleaved protein .Western Blot (Anti-His) analysis of the supernatant fraction.

Figure 3.6 shows the relative size and disposition of the tags before and after thrombin cleavage. Cleavage with the Thrombin protease results in the generation of two fragments (~ 40 and 26 kDa) corresponding to the molecular weight of the free CB2 and the free GST tag respectively (Figure 3.6A). The presence of cleaved and uncleaved protein in the cleavage reaction was verified by immunoblotting with anti-His, anti-CB2 and anti-GST antibodies. As shown in figure 3.6B, probing with anti-His and anti-CB2 revealed bands at 40 and 66 kDa corresponding to the cleaved and the uncleaved fused CB2 receptors respectively. Anti-GST western Blot showed bands for the uncleaved parent protein at 66 kDa (Figure 3.6B).

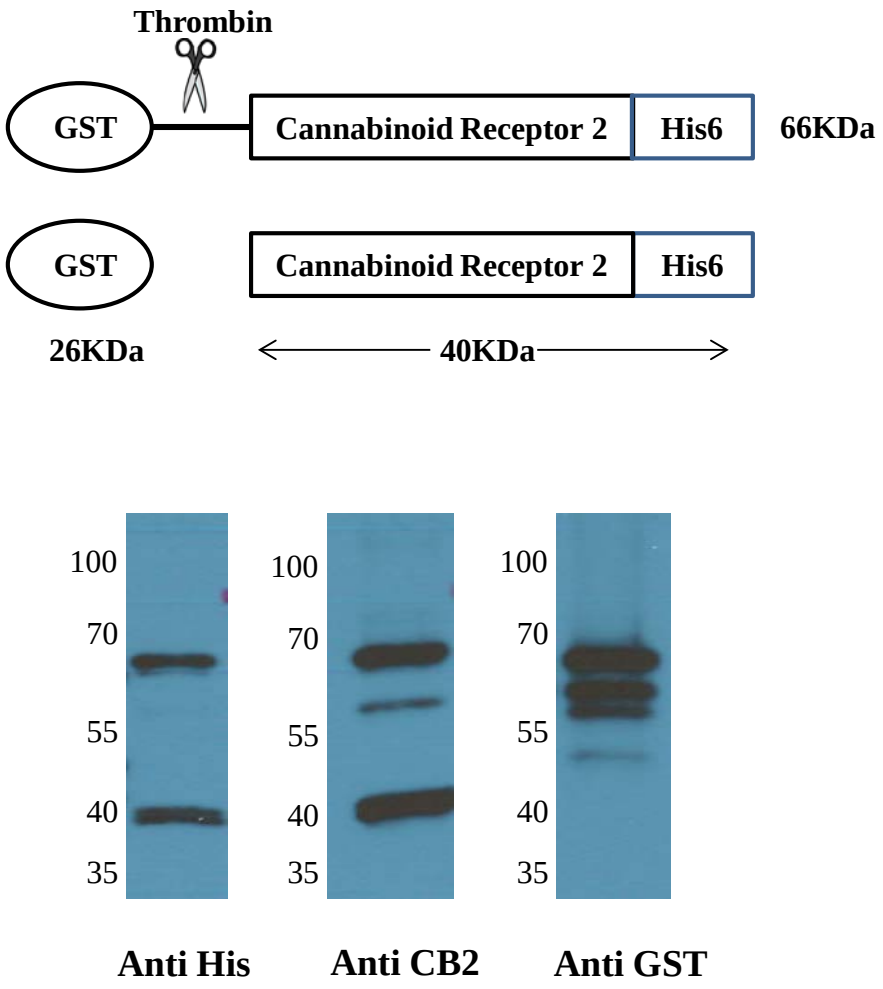

Figure 3.6. Thrombin cleavage of the GST-CB2 fusion protein

(A) Cartoon showing the overall disposition of the fusion protein with the location of the GST fusion partner, thrombin cleavage site and the CB2 receptor in the uncleaved fusion construct (66KDa). Cleavage with thrombin yields two fragments – GST ~26kDa and the His tagged CB2 receptor ~40kDa. **(B)** Products yielded after the cleavage were verified by Western blotting and was probed against Anti-Histidine, Anti-CB2 and Anti-GST antibodies.

For a large scale purification from 8 liters of bacterial culture, the steps in purification was modified to a little extent. A flow diagram of the steps involved and the results are depicted in figure 3.7. A complete one step IMAC procedure was designed and implemented for binding the protein to the column, followed by “on-column” buffer exchange and thrombin cleavage and elution of the bound GST-CB2 fusion protein. Figure 3.7 shows the chromatogram for the combined process. Solubilized GST-CB2 in 1% Sarkosyl was loaded to a His Trap column charged with Ni^{2+} (Figure 3.7, step A) and the column was washed to remove unbound and non-specifically bound materials (Figure 3.7, step B). Buffer was exchanged on-column for 20CV (Figure 3.7, step C) prior to the cleavage step. The large amount of the buffer passed through the affinity column binding the protein ensured the complete “on-column” buffer exchange. This allowed for the complete replacement of 1% Sarkosyl with 0.1% Dodecyl maltoside (DDM). Next, 1000 U of thrombin protease was injected into the column and incubated overnight (Figure 3.7, step D). The cleaved protein was eluted from the column by washing the column with the thrombin cleavage buffer supplemented with 500mM imidazole (Figure 3.7, step E).

Our small scale batch experiments have indicated that the GST can bind to the glutathione ligand (GST Trap Sepharose) while in the thrombin cleavage buffer and effectively allow the separation of the uncleaved GST-CB2 and the free cleaved GST from the cleavage mixture. Using this knowledge a clean and charged GST Trap (GE Healthcare) column was used in the step of the IMAC procedure. The column was attached beneath the HisTrap as the bound CB2 (and also the uncleaved GST CB2 and the associated GST) was eluted from the HisTrap in the Thrombin cleavage buffer.

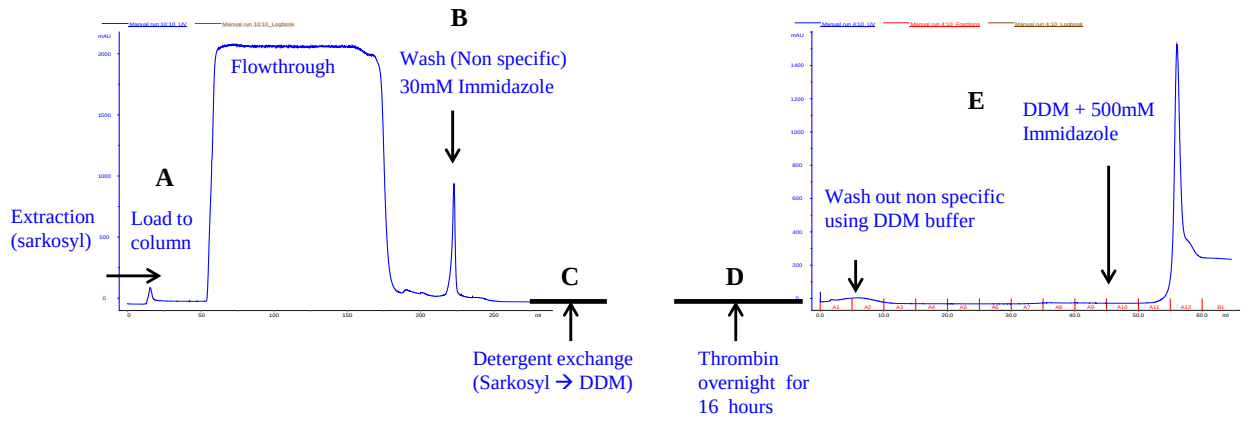

Figure 3.7. Step-wise purification, detergent exchange and on-column thrombin cleavage of GST-CB2 fusion protein

Multistep purification and on-column cleavage process was designed for the expression purification and cleavage of the CB2 receptor. **(A)** The GST CB2 extracted in 1% Sarkosyl was loaded to the HisTrap. **(B)** Unbound and non-specifically bound proteins were eluted by washing the column with loading buffer supplemented with 50mM Immidazole. **(C)** Following this, HisTrap column was washed with 20 column volumes (CV) of the thrombin cleavage buffer (DDM). **(D)** Thrombin 1000U was injected to the column dissolved in cleavage buffer (1CV) and was incubated for 16 hrs at RT. **(E)** Column was connected back to the pre-equilibrated system and the unbound non-specific proteins were eluted followed by the elution of cleaved CB2 receptor in thrombin cleavage buffer supplemented with 500mM Immidazole.

The eluted fraction from the IMAC procedure was was cleaned by Zip Tip and next subjected to MALDI TOF mass spectrometry. MALDI TOF analysis with the eluted cleavage mixture (obtained post affinity chromatography) (Figure 3.8) showed the presence of uncleaved parent GST-CB2 (~ 62.3 kDa), cleaved CB2 (~38.1 kDa) and free GST (~26kDa). The high amount of noise in the spectrum was contributed by the presence of detergents in the cleavage buffer in the eluted sample. However, MALDI-TOF analysis of the eluted fraction revealed presence of all the three entities in the mixture population. Thus, we found moderate to no improvements in the removal of uncleaved CB2 and the free GST fragment by using the GSTTrap in line with the His Trap column while elution. This inefficiency can be attributed to the fact that the interaction between the glutathione ligand and GST is relatively slower and the GST Trap is unable to bind and remove the contaminants at a flow rate of 3 ml/min in which the protein was eluted.

Next, size exclusion chromatography was conducted to further separate out and purify the cleaved CB2 from the cleavage mixture. SEC chromatogram obtained with the cleavage mixture resulted in separation by overlapping peaks when separated through 30ml Superdex column. However the cleaved CB2 (MW~35kDa) was isolated clearly in the fraction 5 of the chromatogram (Figure 3.9A). The presence of the cleaved receptor in the fraction 5 was verified by SDS-PAGE and CBB staining (Figure 3.9B). To determine the presence of higher order structures it was important to separate the proteins by native PAGE. This allowed for the visualization of the higher order structures and their relative amounts in the gel. The protein obtained from the SEC showed a single band in a native PAGE gel confirming the absence of any higher order structure formation (Figure 3.9C). This sample was then exchanged to the mildly denaturing buffer which serves as an ideal starting point for refolding procedures.

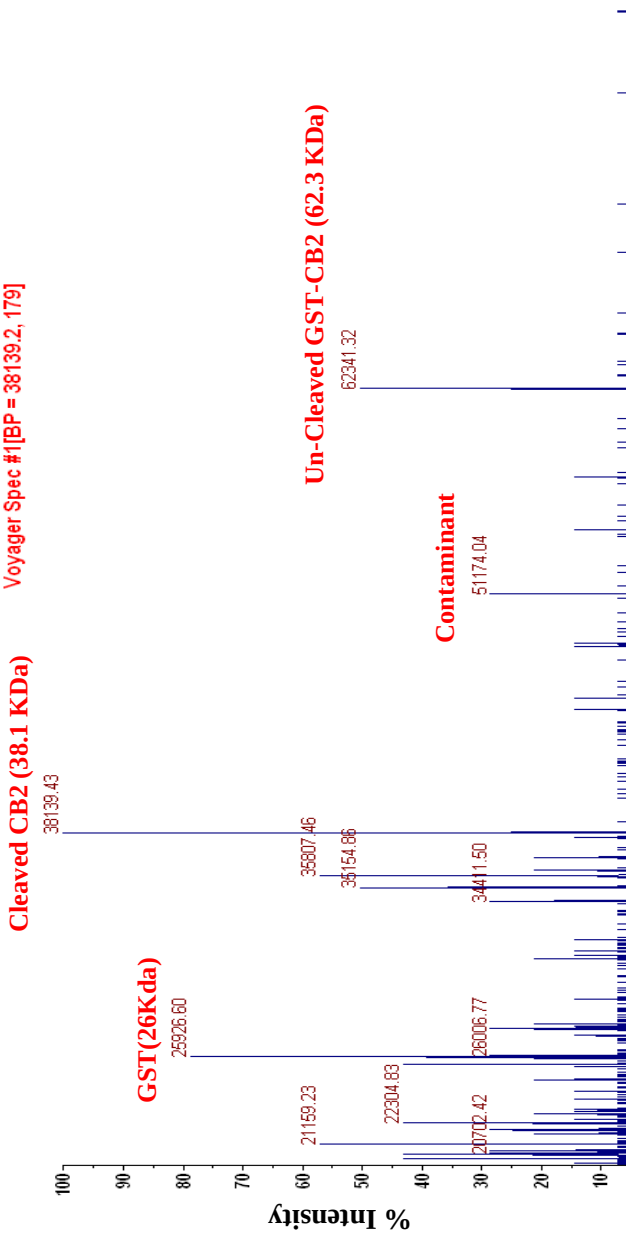

Figure 3.8. MALDI-TOF mass spectrometry analysis of the cleavage mixture.

Protein eluted from the IMAC column following cleavage was composed of the uncleaved parent protein, cleaved protein of interest and the free GST. This ternary mixture is in the buffer containing detergent Dodecyl-beta D maltoside (0.5%). The detergent removal was attempted by using a 10µl Zip Tip (Millipore). Zip tip was pre-equilibrated with the mobile phase for the mass spectrometer (70% Acetonitrile, 30% Water). Clarified protein were spotted on a MALDI plate and the spectra was acquired in a Voyager MALDI-TOF in linear positive mode. Results show the presence of the three components and a high background noise due to the presence of detergent DDM.

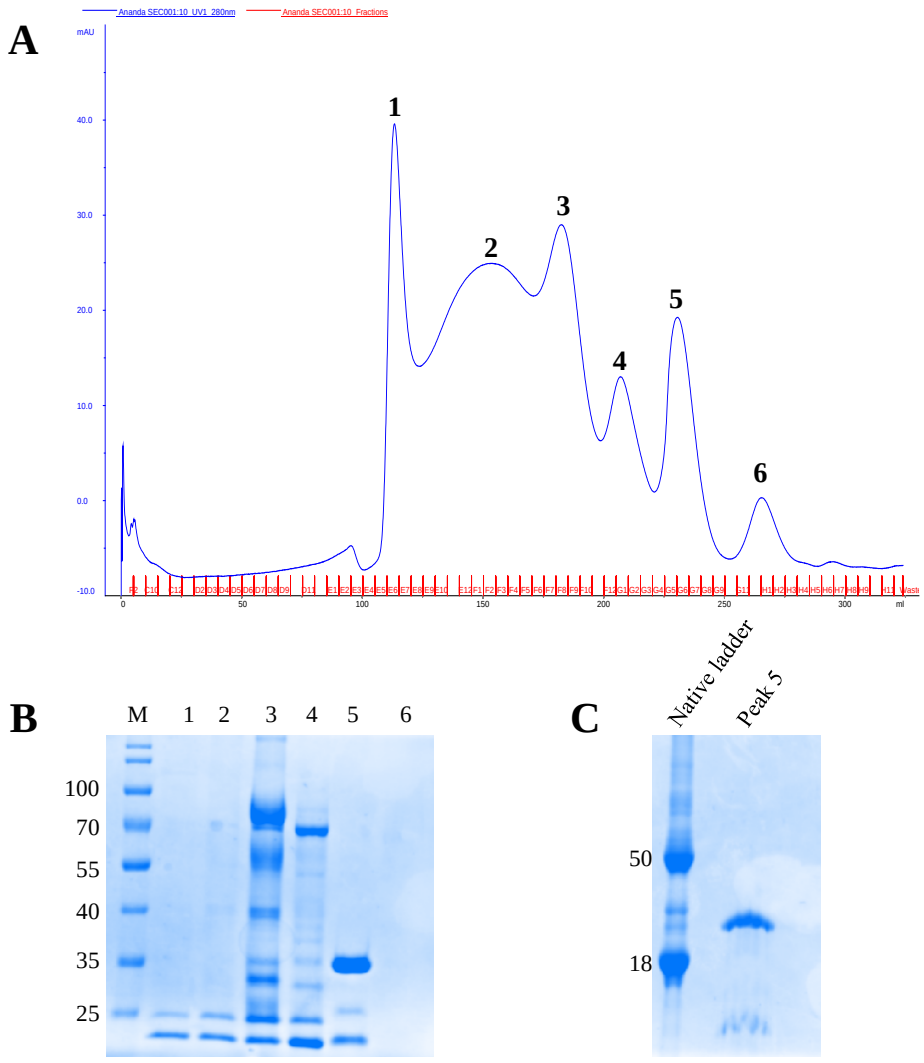

Figure 3.9. Size Exclusion Chromatography (SEC) and Detergent exchange of protein post cleavage.

Eluent protein from the IMAC column comprised of a mixed population of the parent CB2, cleaved CB2 and the free GST. These were separated by SEC using a Superdex FPLC column. **(A)** Chromatographic profile showing the presence of several higher order structures along with the protein of interest (POI). **(B)** POI was eluted under the "peak 5" as seen by the SDS PAGE CBB staining. SEC additionally helps for buffer exchange from 0.1% DDM to 0.5% SDS. **(C)** Proteins were separated by a Native PAGE and were stained by the CBB stain. No higher order structures dimers/ monomers were noticed.

3.3.3 General Discussions

The GST expression system and the thrombin protease offers several advantages compared to the TrpΔLE expression system. Firstly the GST fusion tag serves both as an expression enhancer and a tag which can be used for the purification even in the presence of detergents. However maintaining a non- denaturing condition is very important while carrying out GST tagged protein purification.

To enhance the expression and yield of the protein of interest, it is imperative to ensure that the bacterial cells can transcribe the exogenous gene and thus express the recombinant protein to the maximum efficiency. Codon optimization of the sequence of the structure gene is a very important parameter to aid in the efficient expression of the "payload" protein. The tRNA's which suffer from the rare codon mismatch problem between the eukaryotic and the prokaryotic systems include the amino acids Arg (R), Isoleucine (I), Proline (P) and Leucine (L). To account for this discrepancy, either the construct sequence can be codon-optimized or a bacterial cell line capable of superior handling of rare codons can be used for the expression. Several bacterial expression strains have been designed and modified to include the tRNA's coding for the rare codons present in eukaryotic cDNA. Online predictive tools like https://www.idtdna.com/CodonOpt?c=US help determine the amount and the percentage of the rare codons and also generate codon optimized sequences from a starting parent gene.

Fusion of GST tag to the CB2 gene, does confirm some degree of toxicity to the cells. The combination of BL21 cells transformed with pGEX expression vector leads to transcription of the exogenous gene under the control of the *tac* promoter. On the other hand, any DE3 strain of cell would initiate transcription under the influence of the strong T7 promoter. Expression of the fusion construct was hence preferred in a non-DE3 bacterial strain like BL21 cells for

controlled expression of the POI to reduce levels of toxicity. With a similar intention to control the disadvantage of toxicity in cells, we induced the cells at a relatively much higher OD_{600}. This would allow the cells to increase in number (biomass) before induction. Post-induction the cells would not grow as healthily as before, due to the toxicity of the protein expressed. The larger biomass per unit of culture volume could compensate for the lesser amounts of protein produced in the individual cell level. Procedural modifications that support increased protein production by modifying the DNA sequence or decreasing the toxicity of the protein produced in the cell can be implemented together and would synergistically work to improve the expression level of the protein production.

The cell pellet obtained must be processed in a manner to partially purify the protein of interest, or enrich the inclusion bodies. This essentially entails separation of the soluble proteins from the membrane bound proteins (by solubilizing them with Triton X 100). Several hydrophobic proteins tend to associate with the IBs which can be separated by washing them with non-specific detergents. Thus, it is important to resuspend the pelleted material completely in the washing steps.

The protease cleavage is a key step within the purification and absolutely required in a case when the fusion partner protein is relatively large or its removal is important for the proper folding of the receptor protein. This is because the presence of the fusion partner can affect both the physical and chemical properties of the fusion construct e.g. solubility, overall shape, charge state, molecular weight etc. However it is also important to consider what this may mean for the protein of interest before and after the cleavage. For example for a relatively hydrophobic fusion construct cleavage and release of the fusion partner “tag” from the “protein of interest” may result in an enhancement of the solubility of the “protein of interest” and *vice versa*. A

comprehensive review of different proteases and their removal strategies have been listed in the Section 1.1.4. During the process development steps, it is to be noted that we found a steady and relatively high speed of protease injection for “on-column” cleavage is required to spread the protease to all corners of the column homogenously.

Another key factor to be considered while purification and refolding is formation of higher order structures of the purified receptor. These may include dimers, trimers and other oligomers. The propensity of formation of higher order structures is driven by concentration of the monomer and the relative proportion of the monomers:oligomers. To separate the uncleaved proteins and the free GST from the CB2 and also exclude any higher order formation it is extremely important to separate the contaminants (and other higher order structures) by Size Exclusion Chromatography (SEC). Secondly, the Size column can be used as an effective tool for buffer exchange to introduce a relatively more stringent denaturing condition in preparation for the subsequent refolding steps.

3.4 CONCLUSION

Overall inclusion body directed expression of the CB2 receptor can lead to overexpression and purification of the CB2 to homogeneity. In our first approach using the TrpΔLE-Xa-CB2 we were able to express in large amounts and purify the TrpΔLE-CB2 protein to ≥90% purity. However the entire fusion construct was rendered extremely hydrophobic due to the presence of the TrpΔLE fusion partner and purification was carried out in harsh detergent (10mM SDS). Inspite of the fusion protein being stable in the high amounts of denaturing detergents, we were unable to carry out protease cleavage in this high detergent concentration. Lowering the detergent would lead to partial or complete loss of the fusion protein from solution by precipitation. Due to these reasons we switched to the GST tagged CB2 receptor fusion protein IB expression. The GST tag confers greater solubility of the fusion protein, provides additional advantage of protection from proteolytic cleavage and can also be used for affinity purification or removal. Using the GST tagged CB2 we developed a complete on column protein purification, detergent exchange and cleavage procedure. The cleaved CB2 receptor obtained from this procedure was exchanged to 0.5% SDS which was subjected to refolding procedures which will be discussed in the next section.

4.0 REFOLDING STRATEGIES AND BIOPHYSICAL ANALYSES

4.1 INTRODUCTION

4.1.1 Driving forces in protein folding

The first high resolution or atomic level structure of proteins was observed during the 1960s which heralded a new understanding of regularity and orderly arrangement in structures. This led to the speculation about how the primary structure of a protein dictates its folding arrangement and hence it's functional native structure. That the amino acid sequence is necessary and sufficient for complete folding of the protein was shown by Christian Anfinsen and co-workers (Anfinsen, 1973). It was believed since long that folding of protein involves an interplay of several interactions that can be ionic or hydrophobic in nature. However, around the 1980s it was proposed theoretically that folding is brought about primarily by hydrophobic interactions (Figure 4.1). Electrostatic interactions can be ruled out as the proteins have much lesser charged residues on the surface and that these are concentrated near surface patches which have high dielectric constant. Hydrogen bonding plays an important role in the process of protein folding. The hydrogen bonding interactions among the backbone amide and the carboxyl group in a protein is a key component of all secondary structures (α-helical and β-pleated sheets).

However, the backbone C_α-N_α tracing is similar for all proteins. It plays an important role in protein folding but is not a decisive factor since all proteins have similar hydrogen bonding interactions. It is therefore clear that side chains determine the route of folding and that it is mediated by hydrophobic interactions. Several reasons can be suggested to support this statement : (a) A major structural element in proteins is the presence of the hydrophobic core in which all hydrophobic amino acids are sequestered; (b) Computational studies show energy difference and stabilization by 1-2 Kcal/mol for transferring a hydrophobic side chain from an aqueous to an "oil-like" media (Wolfenden, 2007); (c) A non-polar solvent can readily denature a protein; and (d) Jumbled sequences of proteins which retain their polar and non-polar sequences (and no other significant stabilizing force) can fold to their expected native structures in the renaturing environment (Bradley et al., 2007; Cordes et al., 1996; Hecht et al., 2004). Alpha-helical and beta-pleated sheets provide the opportunity to pack up the long polypeptide chain into an orderly arrangement which is reminiscent of an airport security-check waiting line. Studies on "lattice models" and "tube models" of proteins have shown that the overall protein structure is stabilized by the chain compactness which is indirectly controlled by the hydrophobic force to collapse. Hence it is important to understand that protein sequences containing regular repeats or patches of hydrophobic sequences have the ability to fold into native functional structure as long as the correct milieu is available.

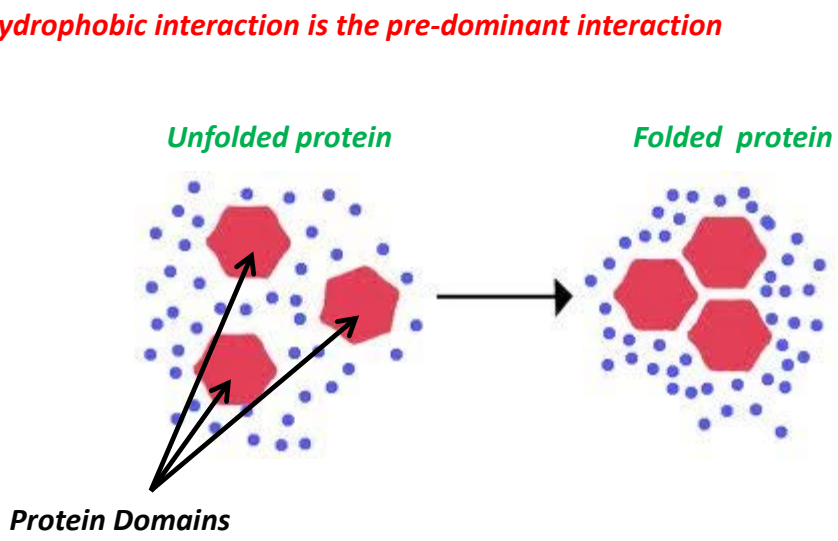

Figure 4.1. Interactions in protein folding.

Individual domains in the protein is brought together from the denatured protein predominantly by hydrophobic interactions. Other interactions like ionic –interactions, hydrogen bonding and Van der waals interactions also contribute to receptor folding.

4.1.2 Membrane protein refolding

Membrane proteins are special cases of proteins in which a reversed arrangement of the hydrophobic and the polar groups have been evolutionarily favored due to their location in the membrane and the presence of the lipid belt region. Membrane protein refolding is convoluted both by their complicated nature and the lack of reproduction of “native lipidic” condition in

which the proteins can refold to functionality. It is to be noted that the process of folding and insertion of the membrane proteins α-helical and β-barrel is not well understood. The precise control of this process is facilitated at various steps including the endoplasmic reticulum (ER) and during membrane insertion. Thus, many studies were directed to the understanding of membrane protein trafficking (Tan et al., 2004). The refolding of Alpha helical proteins will be under consideration due to the alpha helical nature of all GPCRs. Computational biophysical studies have started to provide the theoretical basis (Booth and Curran, 1999) of the membrane protein folding process. The process of studying purified membrane protein refolding is cumbersome due to the low solubility of the membrane protein and lesser shelf time of the starting materials for refolding trial experiments. This is why majority of biophysical studies of membrane protein refolding has been done on the bacteriorhodopsin which is much more stable in a detergent environment than a typical membrane protein and serves as a model system for understanding the principles of membrane protein. A general pathway of two-stage refolding has been suggested for α-helical IMP (von Heijne, 2011). During the first step the transmembrane helices take shape and the extra and the intracellular connectors also start taking up their shape and form. This process is facilitated by the stability of the hydrogen bonding between the backbone amide and the carbonyl group which more stable in the low dielectric environment.

In the second step of the refolding process it is assumed that individual helices come together and organize themselves into the most energetically favorable structure. During this step there is a competition between the propensity of the protein to refold or aggregate. Refolding will occur when the *intra*-molecular interactions will be favored over the *inter*-molecular interaction. It is widely accepted and noticed that increasing the concentration of the unfolded solubilized membrane protein leads to their aggregation and precipitation from the solution phase. In a more

refined theoretical model a third step of ligand binding, folding of the loops and the final formation of the quaternary structure has been proposed (Weik et al., 1998). The balance between aggregation and refolding also largely depends on the refolding environment and an intricate balance must be reached between the “too harsh” and “too mild” environments. To determine the correct condition different parameters like pH, salt, protein concentration can be changed. However the composition and amount of the detergent, detergent-lipid or lipid in the refolding environment always poses as the most crucial factor.

4.1.3 Current refolding strategies of GPCRs from inclusion bodies

The process of refolding is arrived at after the high level expression, solubilization and purification of the GPCR IBs. Refolding strategies may be classified into two pathways depending on the nature of the stabilization environment; the traditional detergent-lipid pathway and the amphipol or proteic pathway (Figure 4.2). The approach to each of these pathways has been described in the next page.

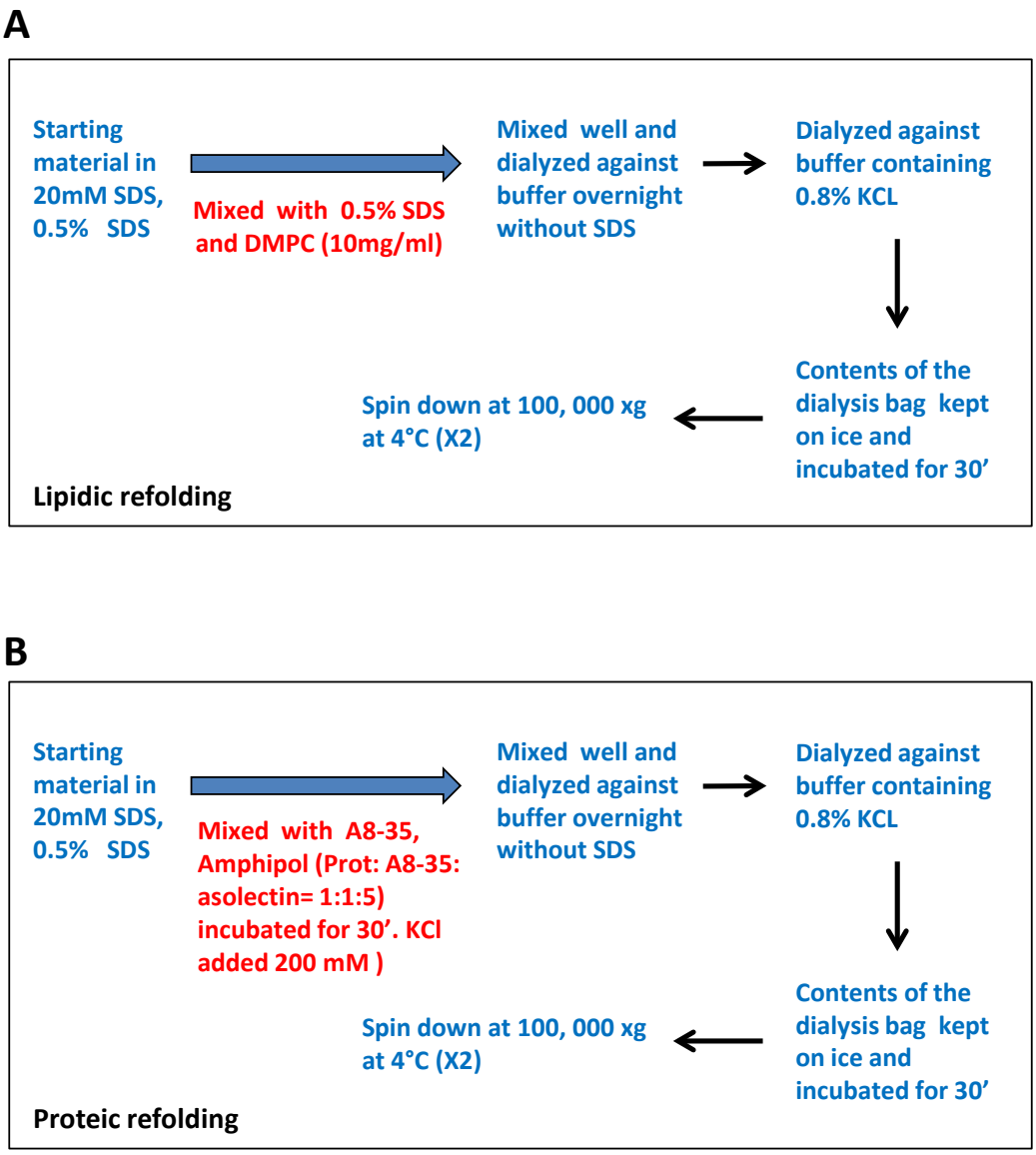

Figure 4.2. Schematic diagram representing the pathway of refolding the CB2 by "lipidic" and "proteic" pathways.

Both methods require the cleaved CB2 receptor in the denatured condition (0.5% SDS) **(A)** Lipidic refolding pathway requires the generation of the CB2, SDS and DMPC ternary complex and the controlled removal of the SDS by dialysis to provide lipid enrichment. **(B)** Amphipol mediated refolding was carried out by the rapid removal of the SDS by KCl precipitation.

It is to be noted that almost all membrane protein including GPCRs retain significant amount of alpha helical (or secondary structure) content in SDS (Miller et al., 2009; Muller et al., 2008). About 40% alpha helicity was retained in the μ-opoid receptor at the pH of 7-8 in a 0.1% SDS solution (Muller et al., 2008) which is slightly less than the total alpha helical content in the fully functional receptor. All these studies taken together suggest that the SDS-solubilized receptor is "partially prefolded" and this "not-harsh-enough" does not completely unfold the protein of interest. Initiation of folding from the SDS-solubilized receptor has been undertaken by the two popular alternative approaches using "lipidic/DMPC" and "proteic/A8-35" stabilization matrices.

4.1.3.1 Refolding the GPCRs in lipidic matrix

Refolding the denatured SDS-solubilized GPCR in a membrane mimetic base seems to be the most rational strategy. Although the native eukaryotic membrane has a precise composition of various lipids, a similar environment can be generated by amphipathic molecules including but not limited to detergents, lipids, detergent-lipid mixtures, bicelles and lipid vesicles. The leukotriene receptor 1(BLT 1) was refolded in 30% Lauryldimethylamine-N-oxide (LDAO) (Baneres et al., 2003) and the BLT2 was refolded to its functional state in n-dodecyl phosphocholine (DPC): hexadecyl-β-D-maltoside (HDM) mixtures (Arcemisbehere et al., 2010). It was also found that the addition of asolectin improved the percentage of functional recovery in both the cases. In a more recent study the human parathyroid hormone receptor 1 and the mouse CB1 were refolded in the non-ionic detergents DDM and Cymal6. Similarly the olfactory receptor OR5 was solubilized in Sarkosyl, refolded within the non-denaturing detergent digitonin and exchanged to lipids like the POPC / POPG mixtures.

4.1.3.2 Refolding GPCRs in Amphipol

Amphipols are synthetic alternatives to mild detergents and have successfully shown stabilizing effects for the refolding of GPCRs in denaturing environment. Amphipols have been defined as “amphipathic polymers”. It was noticed that integral membrane proteins are generally much more stable in amphipol environment and such stabilization assists in folding the GPCRs to the native state. The prototypic Amphipol A8-35 have demonstrated stabilization role for several GPCRs. Conditions for refolding in Amphipol that were initially developed for the bacteriohodopsin were applied to six different GPCRs like Leukotriene B4 receptors (BLT1, BLT2), serotonin receptor 5HT4A, CB1, ghrelin receptor (GHSR1a) and the vasopressin V2 receptors (Baneres et al., 2011). Amphipols provide a very dependable environment in which there is a high probability of newer GPCRs to be refolded. Further, GPCRs which showed functional reconstitution within the lipids showed much better functional activity within the amphipols. However the refolding of denatured GPCRs in a new environment is case specific (Figure 4.2 B).

Table 4.1. Comparison of GPCR folding yields obtained in different surfactants Adapted from (Baneres et al., 2011).

Receptor	**Surfactants used**	**Average maximum folding yield (%)**
BLT1	Detergent-lipid mixed micelles (LDAO-asolectin)	30
	A8-35	50
	A8-35-asolectin	65
BLT2	Detergent-lipid mixed micelles (DPC-HDM-asolectin)	4
	A8-35	50
	A8-35-asolectin	70
CB1	Detergent-lipid mixed micelles(Fos-choline-16-asolectin)	0
	A8-35	30
	A8-35-asolectin	40
	Detergent mixed micelles (DDM-Cymal 6)	30
5-HT4A	DMPC-CHAPS bicelles	25
	A8-35	30
	A8-35-asolectin	60

4.2 MATERIAL AND METHODS

4.2.1 Refolding the CB2 in lipidic stocks

The procedure involves denaturing the CB2 with SDS and then controlled removal of SDS after introducing the refolding lipid. The lipidic matrix was prepared by dissolving dessicated powder of 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) (Affymetrix) in the SEC buffer (20mM Hepes, 250mM NaCl, 0.5% SDS, pH-8.0) to get a final phospholipic concentration of 10mg/ml. The receptor protein obtained from the SEC was mixed with the phospholipid solution gently. The Protein:SDS:DMPC complex was mixed together and incubated for an hour to allow for complete equilibration of the reaction mixture. Following the incubation, the SDS was removed in a controlled fashion. Two step dialysis procedures were performed – in the first step, the reaction mixture was dialyzed overnight at room temperature against refolding buffer (20mM HEPES, pH-7.3). Dialysis was carried out with the protein in a Slide Lyzer cassette against about > 200 folds of the refolding buffer. Next, the buffer was exchanged to a fresh refolding buffer supplemented with 20mM KCl and dialysed for an additional 6 hrs at RT. The SDS in the ternary mixture was precipitated by the co-incubation with the KCl. The contents of the dialysis bag were transferred into prechilled polycarbonate tube to facilitate further precipitation of the SDS. The precipitated SDS was removed by high speed centrifugation (50,000xg, 30mins, 4°C). The protein concentration was determined by BCA assay. A quick snapshot of the procedural steps to refolding is illustrated in Figure 4.3.

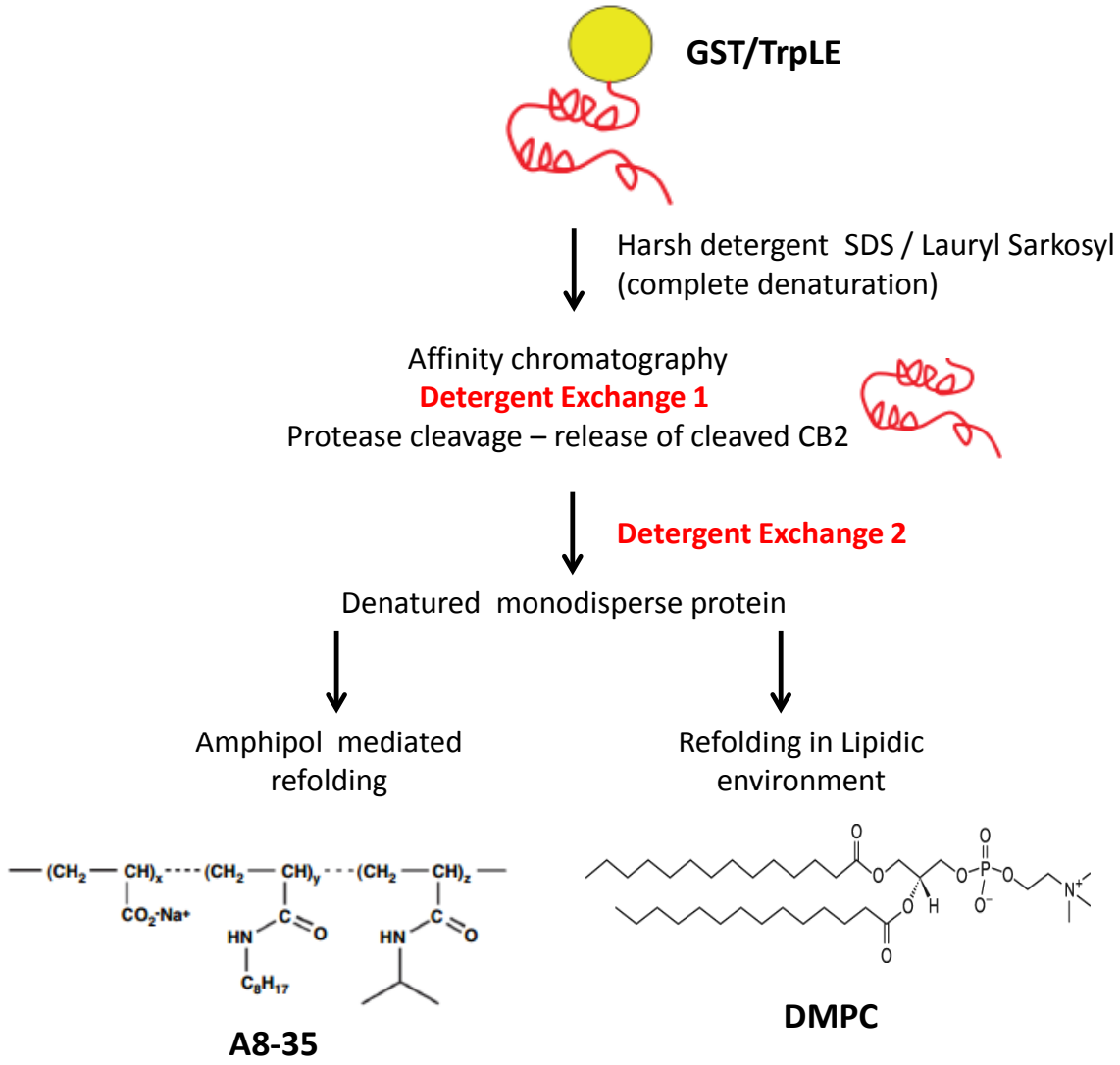

Figure 4.3. Overall methodology involved towards purification and refolding of the denatured- fused CB2 from inclusion bodies.

4.2.2 Amphipol assisted CB2 refolding

The purified protein (in 0.5% SDS) was mixed with Amphipol A8-35 (Affymetrix) and Asolectin (a combination of Phosphatidylcholine, Phosphatidylethanolamine, Phosphatidylinositol, Phosphatidylserine) (Sigma Aldrich) in the ratio of 1:1:5 (by weight). The quaternary complex was incubated for 1 hour. Following this, the SDS removal was initiated by the addition of 150mM excess KCl (pH 8.0). The KCl solution was added rapidly in one shot to the reaction mixture to obtain a final concentration of 200mM. The solution system was mixed well gently and incubated at RT for 1 hour. The precipitated SDS was removed by high speed spin (50, 000 rpm, 30 mins 4^{o}C). The supernatant was dialyzed overnight against 20mM Potassium Phosphate, 200mM KCl pH 8.0, followed by a fresh change of the dialysis buffer and an additional 6 hour dialysis. The contents of the bag were transferred into a prechilled polycarbonate tube and incubated on ice for ~15 mins to facilitate further precipitation of any remaining SDS. High speed spin (50,000xg, 30mins, 4°C) and incubation on ice was repeated twice for the complete removal of the SDS. Supernatant material was collected, protein concentration analyzed by BCA assay and saved at 4°C for characterization. Characterization was done by ligand binding capability of the reconstituted receptor protein.

4.2.3 Radioligand Binding assays

Radioligand binding was performed to determine the percentage of specific binding of a ligand to the purified refolded receptor. Saturating amounts of ligands were used in the Saturation Binding Assay while the Competitive ligand displacement assay measured the capability of one ligand to displace the other for binding to the receptor.

Receptor Saturation Binding Assay

In a "U –bottom" 96 well plate, buffer base and assay components were added in the order as mentioned below. Incubation buffer (InB) (50mM Tris HCl, 2.5mM EGTA, 5mM $MgCl_2$, 1mg/ml BSA, pH 7.4) was added in a volume of 90ul per well for a 200ul assay mixture (80ul in wells for measuring the non-specific binding). The ^{3}H-CP 55,940 was diluted through a range of concentrations by using the Compound Dilution Buffer (CDB)(2mg/ml BSA, 2.5ml TME, 2.5ml of DMSO, 4ml of 2.5% Methyl Cellulose and 3.5 ml water/ 25ml of the CDB buffer). A 10X TME buffer stock solution was prepared (by adding 27.5 grams of Tris Base, 43g of Tris HCl, 9.51g EGTA, 10.165 g $MgCl_2$, pH 7.4). The radioligand ^{3}H-CP 55,940 (Research Triangle Institute, NC) was diluted in the CDB upto a final concentration of 4054.3nM. Dilution was performed by using the equation

*Concentration of Radioligand (M) = radioactivity concentration (mCi/ml)/ [specific activity (Ci/mmol)*1000 (mCi/ml)]*

Saturation Binding assay was performed in triplicate to determine the Total and the Non Specific binding respectively. The Specific binding is the difference between the Total and Nonspecific binding. In triplicate wells designated for non-specific binding measurement, 10µl of 100µM cold ligand (cold CP 55,940) was added to a final concentration of 5µM. 10 µl of the ^{3}H-CP 55,940 was added to eight wells over a concentration range of 50 – 400 pM. Protein samples (proteoliposomes DMPC/A8-35) were added upto a final concentration of 300 pg/well diluted in 100 µl. Components in the wells were mixed gently and incubated at 30°C for 1 hr with gentle shaking. Following incubation the assay mixture was harvested by a Filter Mate Harvester (Perkin Elmer) into a 96 well GF/B plate (pore size 1.0 micron). The filter plate was dried overnight. Next, 30µl of Micro Scint liquid (Perkin Elmer) was added to each well of the

GF/B plate and incubated in dark for 15 minutes. The counts per minute from the wells were read in a Top Counter reader.

Competitive Binding Assay

Competitive Binding Assay was performed in the similar 96 well plate format. Several buffers used in the Saturation binding namely the Incubation Buffer, Compound Dilution Buffer are identical in the assay. The compound powders were dissolved in 100% DMSO and were diluted to concentrations ranging from 0.01 nM to 1.0 mM. Selection of eight points for the assay and ligand dilution was determined such as the Ki value is the closest to the midpoint of the dilution range. The exact concentrations of the proteoliposomes do not significantly matter and assays were conducted with 300- 2000 pg/well. Results and the effects of changing the proteoliposomes amounts per well will be discussed in the following section. Assay components were added in the 96 well plate in the order of incubation buffer (30ul), cold competing compound (20ul), 50 ul of the ligand 4nM final concentration. Proteoliposomes were added in the last step. Liposomes or CB2-APol mixtures were diluted in the refolding buffer and the KP buffer respectively. During the process of preparation of the liposomes and final dilution, care was taken to make a homogeneous mixture and the same amount of proteoliposomes was added to all the wells. The final assay mixture (200ul) was mixed well and the plate was incubated at 30°C for 1 hr with gentle agitation in an orbital rotor. Following the incubation step the plate was harvested, dried and read from the GF/B as mentioned for the Saturation Binding assay in the previous section.

4.3 RESULTS AND DISCUSSIONS

4.3.1 Purification and conditioning the CB2 for refolding processes

The correct processing of the cleavage and detergent exchange steps are necessary for the successful refolding to take place. If the starting material of the refolding experiment is not in the correct disposition then the refolding process can fail. The correct conditions include but are not limited to several conditions as discussed below. Homogeniety of the protein sample is perhaps one of the most important factors to get the protein to the correct refolded state. The Size exclusion chromatography (SEC) steps works in the same lines to further polish the eluted protein. SEC chromatogram obtained with the cleavage mixture resulted in separation by overlapping peaks (Figure 3.9A).

4.3.2 DMPC assisted refolding

Proteoliposomes were prepared by the process as discussed above and is shown by the schematic Figure 4.2A. The DMPC lipids provide and act as a surrogate environment for the refolding of the protein (Figure 4.4A). Since the refolding process is a complicated interplay between several conditions, it is difficult to critically evaluate method design and end-point correlation. To initially determine the presence of active receptor, competitive binding was carried out. Inverse agonist SR 144528 displaced 3H-CP 55,940 with a Ki = 4.74 nM (EC50 = 6.47nM , Kd = 2.73) (Figure 4.4B).

However large error bars were noted at lower concentration ranges of the cold ligand which may be probably attributed to irregular washout of the proteoliposomes. Saturation

binding assay was conducted with the refolded receptor. It displayed a trend of saturation for Total and Specific Binding (Figure 4.5). Bmax values for Total and Specific binding were 114576 and 38486 CPM respectively. CP 55,940 exhibited a K_d of 18.47 in the DMPC refolded CB2. Non Specific binding was determined to be ≥ 60% of specific binding. Known CB2 ligands binding to the DMPC refolded receptor were conducted by displacing ^3H –CP 55,940 with CP55,940 (unlabelled); SR 144528 and PY 2-64 (Figure 4.6). Assay was performed in duplicate (n = 2). Data represented as mean ± SEM. All ligands including agonist, inverse agonist displayed competitive displacement. CPM values obtained after repeated experiments with agonist WIN 55212-2 displayed a high degree of variability however a clear binding trend was observed showing ligand displacement when data was plotted with single point (n=1) values (Appendix A Figure 4.13). All these data suggests that we have designed and generated a very promising method for the in-vitro refolding of the CB2 within the DMPC.

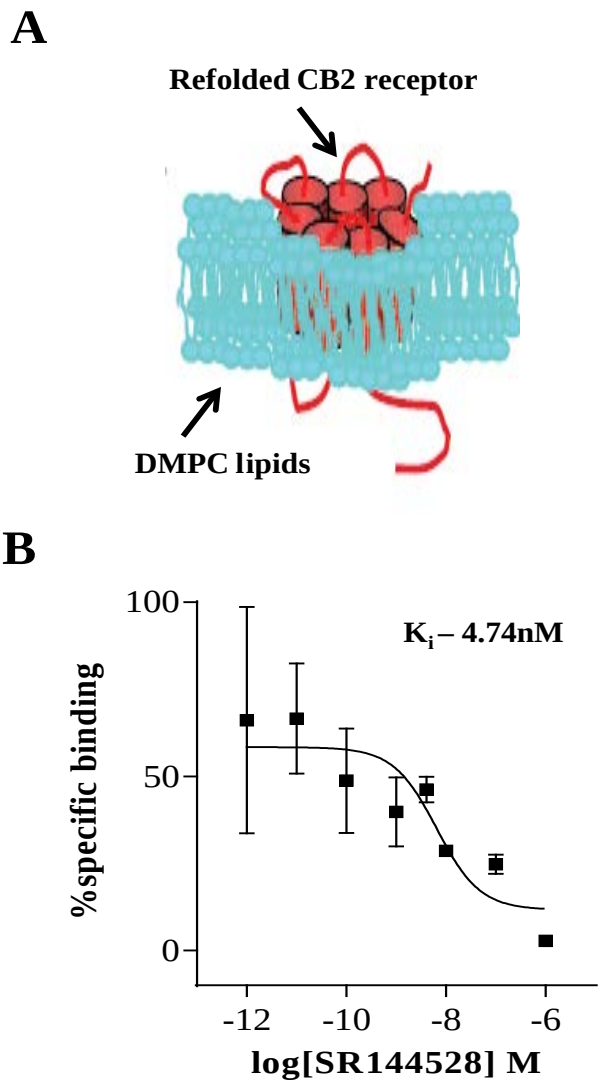

Figure 4.4. Competitive ligand displacement assay with DMPC mediated refolded CB2.

(A) Cartoon showing CB2 stabilized by DMPC lipidic environment. *Adapted from Baneres et. al. 2011.* **(B)** Competitive displacement of the ^{3}H-CP55,940 was obtained by using an increased amount of cold ligands. Binding profile of the inverse agonist, SR 144528 by displacing the ^{3}H CP55940, K_i = 4.74 nM.Assay was performed in triplicate (n = 3). Data represented as mean ± SEM.

The process of dialysis has limiting or cut-off efficiency. As per our observations, it is thus important to remove the residual SDS by adding KCl to the dialysis buffer. This will lead to the precipitation of the SDS by the formation of KDS (Potassium Dodecyl Sulphate) which is insoluble. The increased precipitation of the SDS can be visually seen within the dialysis bag. Following dialysis, it should be a priority to remove the remaining SDS by centrifugation. This process can be facilitated by removing the protein from the dialysis set up and incubating on ice. Following this the proteoliposomes can be separated from the precipitated SDS by very high speed centrifugation. It should be noted that no exact ratio of the monodisperse protein: DMPC solution in 0.5% SDS is available and thus, the lipids are used in much excess.

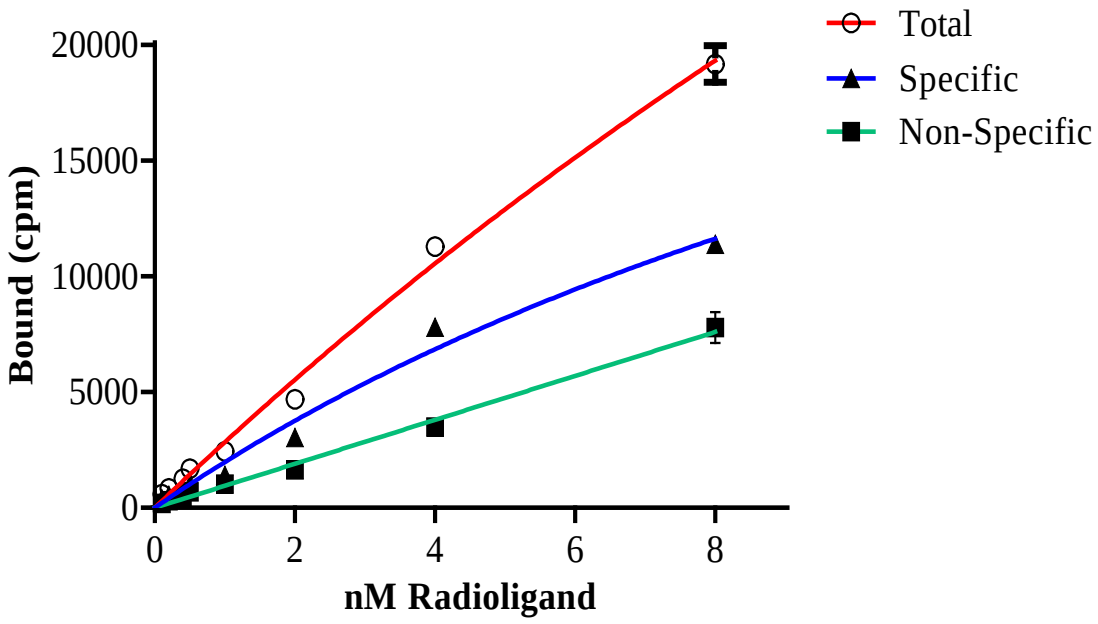

Figure 4.5 Saturation Binding Activity of DMPC mediated refolded CB2

Saturation binding assay was performed with membrane fractions by increasing the concentration of agonist, ^{3}H CP 55940 using a fixed amount of target protein. A 1:1000 excess of cold CP 55,940 was added in the reaction mixture to account for non specific binding. Total (○) and non-specific (■) binding was measured and the deduced specific binding saturation isotherm (▲) was obtained as the difference between total and nonspecific binding. Assay was performed in triplicate (n = 3). Data presented as mean ± SEM.

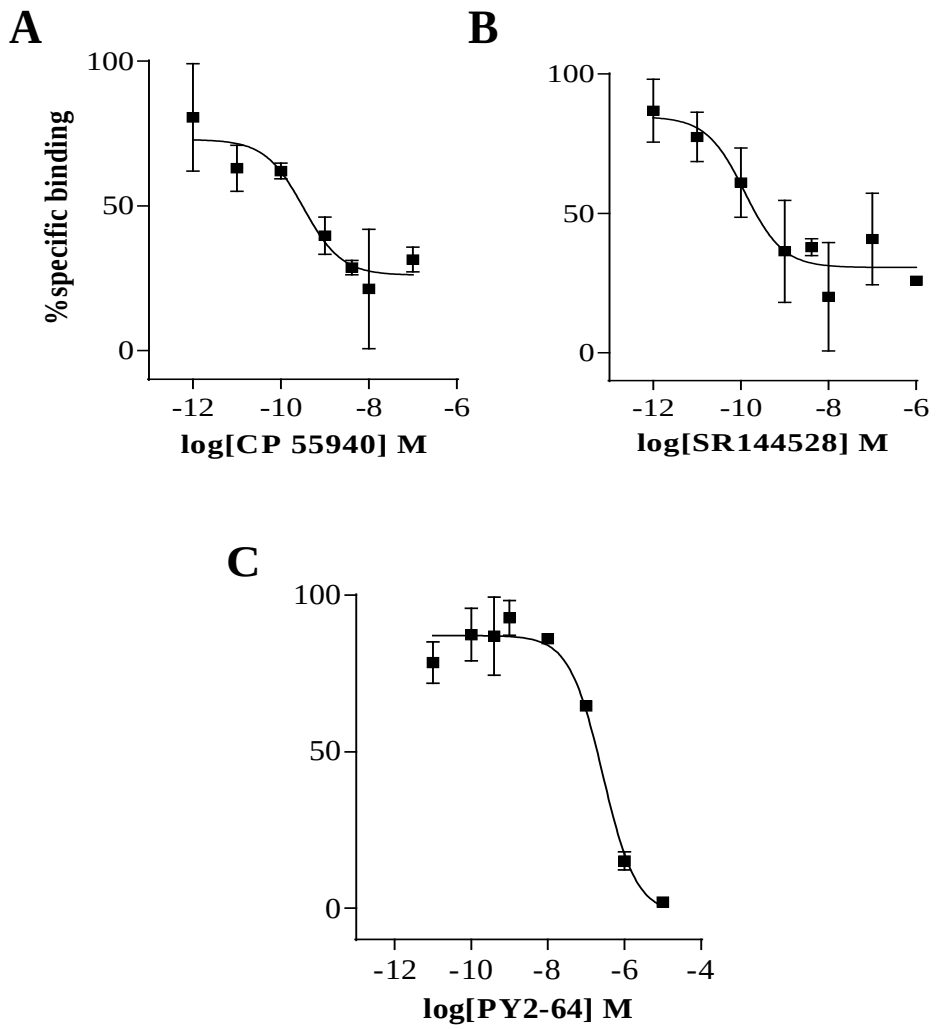

Figure 4.6. Competitive Ligand Binding Activity of the CB2 receptor stabilized within the DMPC lipids.

Competitive displacement of the ^{3}H-CP55,940 was obtained by using an increased amount of cold ligands. Binding profile of **(A)** CP55,940 (unlabelled); the inverse agonists, **(B)** SR 144528 and **(C)** PY 2-64. Assay was performed in triplicate (n = 3). Data represented as mean ± SEM.

4.3.3 Amphipol assisted refolding

Amphipol mediated CB2 refolding was carried out as discussed and is shown by the schematic Figure 4.2B. Initial competitive ligand displacement assays performed on the A8-35 mediated refolded CB2 (^{3}H CP 55,940 displaced by SR 144528) displayed some degree of displacement of the radioactive ligand (Figure 4.7B). However, the absolute CPM values were in a very small range and the error margins on the data points were large. Nevertheless the K_i value obtained for the binding of SR 144528 was 6.97 nM which is well within the range of the K_i values reported for the SR compound (with CB2 receptors expressed in transfected mammalian cell lines). Binding was also conducted on separate preparations of CB2 refolded in A8-35, however no typical ligand displacement curves were generated (Appendix A Figure 4.14). This may be due to the incompatibility of the CB2 with the particular amphipols system (A8-35:Asolectin combination). Interestingly, the CB1 receptor displayed a decent amount of refolding and activity with the A8-35 asolectin (Dahmane et al., 2009).

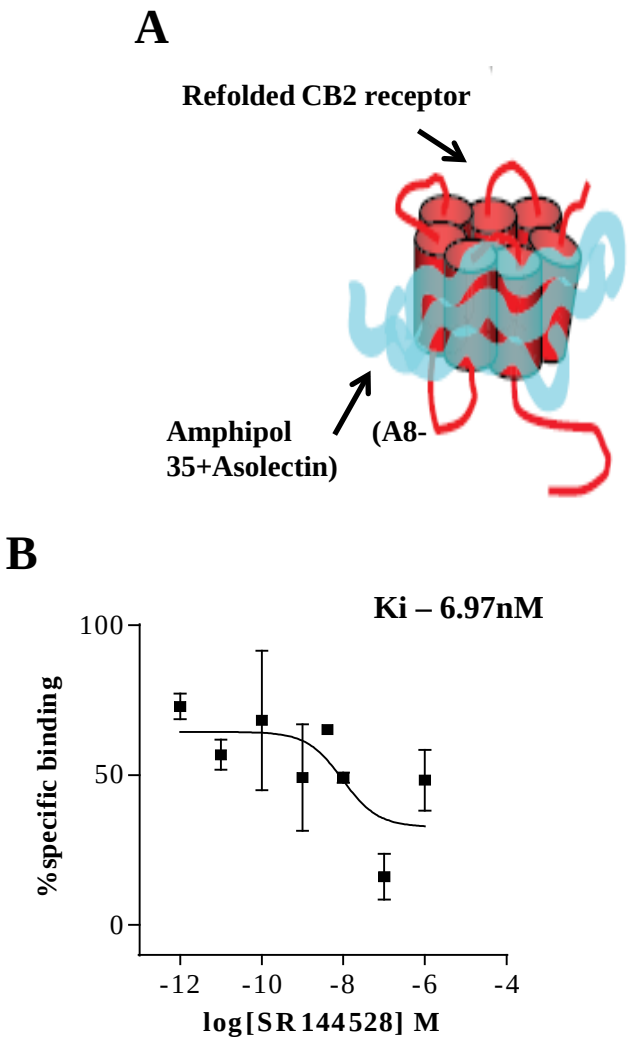

Figure 4.7. CB2 refolding in Amphipol

(A) Cartoon depicting the stabilized CB2 receptor within a thin layer of the A8-35 amphipathic detergent (also supplemented with asolectin). *Adapted from Baneres et. al. 2011*. **(B)** Competitive ligand binding profile of the ^{3}H CP 55940 replaced by increasing concentration of the cold SR 144528.

4.3.4 General Discussions

Amphipol assisted refolding was developed with the goal of achieving a generalized approach for the folding of membrane protein GPCRs. However in our case we do not see much appreciable ligand binding in proteins that were refolded with A8-35 and asolectin. The Amphipol mediated refolding method works in the same lines as that of the DMPC lipid based method except for that amphipols have a “protein–like” structure and the reaction system have been supplemented with asolectin. Asolectin serves as a stabilization matrix. It has also been shown that the CB1 receptor can be refolded and stabilized even in absence of asolectin. There are some key considerations during amphipol mediated refolding of the CB2 receptor. The starting material CB2 receptor in its unfolded stage must be completely denatured, to go through the process of refolding. This means that the amount of SDS used in the refolding system must be greater than equal to 0.8%. This will ensure that the CB2 is nearly completely unfolded at the start of the refolding process. A big difference in the refolding procedure between the DMPC and the Amphipol mediated refolding is the removal procedure of the denaturant. While the SDS is removed in a slow and steady fashion by dialysis during DMPC assisted refolding, a more drastic method is preferred for the A8-35 mediated refolding. This difference may be attributed to the difference in the rates of association between the hydrophobic receptor core and the DMPC lipid or the A8-35 Amphipol. The rapid removal of SDS (or at least the majority of the SDS present in the solution system) was attained by the rapid addition of KCl in the excess of 150mM to that of the SDS concentration in the refolding mixture. There can be two routes for the generation of the Integral Membrane Protein – Amphipol complex (IMP-APol comp). In some cases if the IMP is

yielded in the native conformation within the detergents then addition of the amphipol will result in the formation of the ternary complex (IMP-detergent – Apol) (Tribet et al., 2009; Zoonens et al., 2007) which can then undergo the process of detergent removal to form intact IMP-APol complexes. In these cases the Amphipol is present as a thin layer covering the hydrophobic surface of the protein (Zoonens et al., 2005).

Testing the pharmacological activity of a refolded receptor provides unique challenges. The reconstituted receptor either in the lipids or in the Amphipol are much smaller in size compared to membrane fragments that are generally used for testing receptor pharmacological activity. Firstly, the amount of the protein that would be required for the assay set up would be much lesser compared to the traditional methods using the membrane fragments. Due to this and the variability of the amount and quality of the protein from batch-batch it is very difficult to determine the exact amount of protein to be added/ well of the assay system and ligand range of the cold compound that needs to be added to provide a larger difference of the Counts Per Minute (CPM) values between the highest and the lowest ligand concentrations. A competitive ligand displacement assay does not have a huge dependence on the absolute quantity of the protein and can be conducted first to probe for the presence of receptor functional activity. Once functional activity has been detected, saturation binding can be conducted to determine the B_{max} and K_d values of the receptor preparation. This order of experimentation is important as receptor saturation might not be observed if saturation radioligand (^{3}H-CP 55,940) concentrations are not reached or if functional receptors are absent. A general guideline for setting up the saturation binding assay from our experience is to start from very low to medium and higher amounts of proteoliposomes per well of the assay system. This will ensure that the receptor amounts are low enough to be saturated with the given highest dose of the radioligand. At this point it may also be

necessary to also increase the amount of the radioligand in addition to decreasing the protein amount. A relatively very high amount of non-specific binding was observed in the case of the DMPC folded CB2 which may be due to either the presence of the non-functional receptors or the presence of contaminating proteins which bind non-specifically to the ^{3}H-CP55,940. For the WIN 55212-2, the binding profile obtained was not conclusive. This may be due to the fact that the WIN 55212-2 being a potent agonist for the CB2 receptor would activate the receptor by the disruption of the intramolecular stabilizing bonds which is stabilizing the receptor structure in the first place. This is consistent with the observation that GPCRs (within LCPs) have yielded many more crystal structures with stabilizing inverse agonist, antagonist than with potent agonists. Furthermore the binding profile of PY2-64 compound synthesized by our group seemed to display high affinity binding with the CB2 receptor refolded in DMPC.

Table 4.2 compares the binding affinities of the CB2 ligands to the native versus *in vitro* refolded CB2 receptor. Binding affinities of CB2 ligands like CP, SR and WIN vary between laboratories and independent experiments. The K_i values for the native receptors from transfected cell-lines are thus derived from the standard values from Tocris Biosciences (http://www.tocris.com/pharmacologicalBrowser.php?ItemId=4983#.UqZKFEAo6M8). We suggest several reasons to account for the difference in the binding affinities of the CB2 ligands between the native CB2 (expressed in the transfected mammalian cell lines) and the CB2 refolded in the DMPC lipids. In overall the standard compounds CP 55,940 (non classical CB2 agonist) and the SR 144528 (CB2 receptor inverse agonist) exhibit binding affinities in the range of (0-10 nM). These two ligands exhibit a 3 fold and 5.55 fold tighter binding with the CB2 in DMPC lipids (when compared to their most potent binding affinities with the native CB2). We would assume that the CB2 receptor is relatively much more unstable within the DMPC than in

its natural membrane environment, although they might have an overall similar structure to exhibit functional ligand binding. The CB2 protein in the DMPC should hence bind more tightly to these ligands to achieve structural stabilization and remain in the most thermodynamically stable state. This should be particularly true in case of the SR 144528- the inverse agonist which in theory should stabilize the receptor in its inactive state. Along the same lines, a potent agonist will lead to the disruption of stabilizing interactions within the receptor and lead to transition from the inactive to the active state. We understand that WIN 55212-2 binding will break the stabilizing interactions in the receptor and lead to structural destabilization or complete structural loss of the CB2 stabilized in the DMPC lipids. Considering the specific receptor saturation binding and the competitive ligand displacement patterns of the CP 55940 and SR 144528 ligands, we can infer that the CB2 receptor refolded within DMPC assumes a native-like disposition. Surprisingly, the inverse agonist PY2-64 bound the DMPC-refolded receptor with a significantly lower binding affinity. A plausible explanation for this phenomenon may be attributed to the difference in the spatial disposition of the key amino acid residues involved in the PY2-64 ligand binding pocket of *in vitro* refolded CB2 versus the native receptor. This explanation however is subject to further biophysical analyses.

Table 4.2.Comparison of K_i values of ligands binding to the CB2 receptor obtained from transfected CHO cell line versus CB2 receptor obtained within the DMPC lipids.

Ligand Name	Ligand functionality	Ki in transected cell line (CHO CB2 membrane fractions)	Ki in CB2 refolded in DMPC
CP 55,940	Non classical agonist	0.69-2.8 nM	0.235 nM
WIN 55,212-2	Agonist	3.13 nM	No Binding
SR 144528	Inverse agonist	0.5-8 nM	0.09 nM
PY2-64	Inverse agonist	0.5 nM	218 nM

4.4 SUMMARY AND CONCLUSION

4.4.1 Process Summary

In the project we started out with the goal of producing purified functional CB2 receptor *in vitro* within stabilizing environments using the *E. coli* as the expression host. Successfully produced protein can be then used for both structural and functional studies. The overall process required three major steps of expression, purification, structural reconstitution *in vitro*. We started CB2 expression with dual fusion tags for membrane targeted CB2 expression using Mistic and TarCF as the N- and C-terminal tags respectively. In spite of functional CB2 expression in the membrane, the system could not be taken forward for the extraction and purification due to the low expression level of the fusion protein. To then avoid the problems arising from the low expression levels of the CB2 receptor, we used IB targeted expression of CB2 receptor. Two expression vectors TrpΔLE-CB2 and GST-CB2 were designed for this purpose. The TrpΔLE – CB2 system had a very high overall hydrophobicity. High concentrations of the detergents were required to keep the fusion protein in solution while there was no or very less Factor Xa cleavage under these conditions. The GST CB2 expression construct provided IBs with higher solubility. Using this system we were able to purify and carry out the process of detergent exchange and thrombin protease cleavage all in one step. Further the cleaved, monodispersed CB2 was obtained after the size exclusion chromatography steps in 0.5% SDS which provided the starting material for the refolding trials. To carry out structural reconstitution or refolding *in vitro* we used Amphipols (anionic polymers). Using standard amphipol mediated refolding procedures we observed no or very shallow ligand binding curves. Better ligand binding was observed using the lipids DMPC. Binding of standard ligand CP 55, 940 (non classical CB2

receptor agonist) was observed by both saturation binding and competitive ligand displacement assays. Furthermore to verify receptor functional activity competitive displacement was carried out with CB2 ligands SR 144528 and PY2-64 (CB2 receptor inverse agonist and neutral antagonist respectively). All ligands displayed classical binding patterns. Binding constant K_i value for the PY2-64 had about 100-1000 fold less affinity from that of the WT CB2 receptor. Using the GST CB2 expression purification system and the DMPC mediated refolding we were able to express, purify and refold the CB2 receptor *in-vitro* to functionality.

4.4.2 Overall Conclusion

The scope of functional expression of GPCRs is enormous and involves many disciplines such as molecular biology, protein biochemistry, protein chemistry and lipid and/or detergent chemistry. Our findings, however limited or case specific, have contributed to all these fields, specifically towards their application to membrane protein biochemistry. GPCR structural biology is in its early stages and high resolution structural and mechanistic information is required for a huge number of receptors which are very important therapeutic targets. This is due to the lack of robust and approachable methods involved in the production of functional GPCRs. Methods are either very costly, resource intensive or are not robust and repeatable. The CB2 receptor, particularly using the inclusion body based approach, has been isolated within lipids in functional form. Both approaches to produce the receptor in its functional form have made significant innovation in terms of vector design and methodology development.

Mistic-CB2-TarCF construct can successfully express the CB2 receptor protein in *E. coli* C43(DE3). The obtained fusion proteins can localize at the bacterial membrane. Importantly, the Mistic-CB2-TarCF fusion proteins show effective binding activity with the known CB2 ligands.

This suggests that the conformational state of the native CB2 receptor, used for specific ligand binding, is retained in the presence of fusion partners. Also, we found that the fusion partners – Mistic and TarCF – in combination, are more effective for enhancing protein expression in *E. coli*, than their use alone. Overall findings from this present study suggest that the targeting of fusion partners to the bacterial membrane is critical to the conformational stability of the expressed CB2 protein. The possible role of the fusion partners for the overexpression and stabilization the CB2 protein is illustrated by the two plausible models (Fig. 2.7) for easy comprehension. In this putative model, the CB2 receptor structure was adapted from the 3D CB2 model reported previously by Xie et. al (Xie et al., 2003), while the structure of Mistic and Tsr (structurally related to Tar) were determined by NMR (PDB:1YGM) (Roosild et al., 2005) and cryo-electron microscopy (Khursigara et al., 2008) studies, respectively. However, confirming the putative model will be subject to further biophysical studies. Currently, we are using the entire fusion protein and microscopy. The trials for 2D crystal generation will be favorably facilitated by the increased molecular weight of the fusion protein complex (Smyth et al., 2003).

In our second approach we used the diametrically opposite approach for the production of functionally inactive CB2 inclusion bodies. A huge impetus for *in vitro* refolding of GPCRs was provided by the development of newer and developed refolding methods and matrices for stabilizing the receptor (Baneres et al., 2011). These methods were implemented in parallel for the greater chances of functional folding.

The overall very high hydrophobicity of the TrpΔLE leader makes it difficult during the isolation and purification of the fusion protein. However the fusion protein had a high level of expression and the protein was isolated by one step pH gradient chromatography. The biggest challenge we encountered with the hydrophobic protein is to carry out protease cleavage. The

protease greatly loses its activity in the presence of the detergents; however the detergents are required to keep the protein in solution. We have always observed a high degree of incomplete cleavage or precipitation of the TrpΔLE-CB2 in a low detergent environment. This meant that in-spite a careful optimization of detergent environment large excess of the Factor Xa protease would be required which would make it very costly approach.

The use of a construct with greater solubility e.g. the GST-CB2 has proved to be a much more useful approach. The GST CB2 can be stabilized in aqueous buffers with very low (upto even 0.05%-in our hands) of very mild detergent like DDM. Furthermore we have also seen significant ability of the GST Sepharose beads to capture and remove GST/GST tagged protein from the solution phase in the low detergent environment. This suggests that the GST tag can indeed be used for both enhancing solubility and purification in IB targeted GPCR expression and purification.

We have in our refolding studies generated receptor refolded within the DMPC lipids. No or very low receptor activity was seen for the CB2 refolding in Amphipol A8-35. Refolding to its functional state depends on the interplay of several physicochemical property of the receptor, the refolding medium and also in the process of SDS precipitation from the refolding mixture.

In conclusion a very attractive method has been established for the production of the functional CB2 receptor which can be taken over for huge number of applications. Figure 4.8 summarizes the overall results from each approach towards achieving our goal of producing functionally active CB2 receptor *in-vitro.*

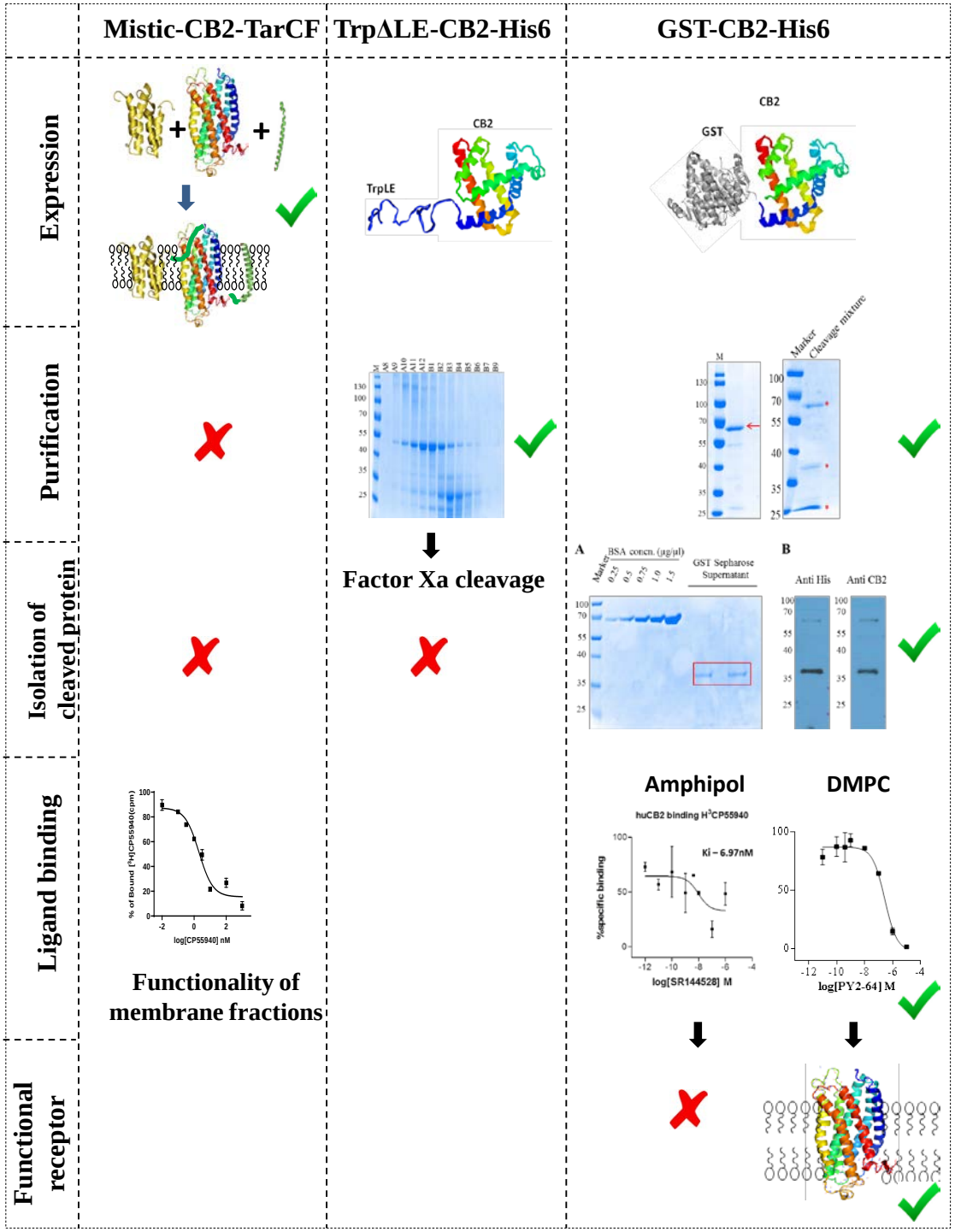

Figure 4.8. Overall summary of results from the three distinct approaches towards producing functionally active refolded CB2 receptor *in vitro*

4.5 FUTURE DIRECTIONS

The future scope of the work carried out can be grouped into two areas. Firstly we will discuss the future scope of this study within the area of methodology development and production of functional CB2 from *E. coli.* This can be correlated to a much broader goal of design and implementation of faster and more robust method for the functional expression of GPCRs from bacterial expression systems. Secondly we will discuss the future studies that may be conducted on the functional CB2 arrived at this method which are likely to be carried out in our / collaborators laboratory.

The development of a robust and dependable method for the functional expression of the CB2 receptor from the *E. coli* will greatly facilitate the structure function studies of the receptor. Firstly an optimized methodology, like we have described, greatly reduce the cost of GPCR protein production. Further *E. coli* offers the ability to produce isotopically labeled protein and exchange among stabilization environments e.g. Lipids to LCP/ nanodiscs for crystallization or receptor dynamics experiments respectively. For similar studies in the future we suggest the adaptation of cyclic denaturation and renaturation steps for GPCR refolding. Other members of the Rhodopsin family GPCRs can be subject to similar solubilization and on column detergent exchange and cleavage steps. As a starting point we suggest the use of DMPC lipids for the refolding strategy however several adjustment need to be made for different GPCRs. As a future study we strongly recommend the use of lipid e.g Asolectin and Cholesterol additives to the refolding mixture. These would perhaps help to attain better stabilized receptor and also determine the role of such additives in receptor stabilization. It would also be interesting to determine the effects of stabilizing ligands (inverse agonist e.g. SR 144528 for CB2) in the refolding mixture. Determination of the stability of the refolded

receptor with time in normal or elevated temperatures to determine receptor thermostability. When working with a different GPCR all steps of expression, purification and refolding experiments reported here should be modified. However, this methodology would serve as a starting point for future studies with other GPCRs.

Functional CB2 receptor obtained within the DMPC may be used for a wide array of experiments. We would like to conduct Cryo EM studies to determine the plausible receptor structure and its disposition within the DMPC lipids. As stated previously the developed methodology can be adapted by simple modification of expression conditions to M9 media to produce uniformly isotopically labeled receptor. Refolded isotopically labeled CB2 may then be used for solid state NMR spectroscopy. With the developments of NMR spectroscopy and data interpretation CB2 dynamics including ligand induced receptor conformational changes can be monitored by solution state NMR spectroscopy. The refolded CB2 also provides a great platform for the Hydrogen-Deuterium exchange studies for the determination of solvent accessible surface area and studies on conformational activation of the receptor.

APPENDIX A

SUPPLEMENTARY FIGURES

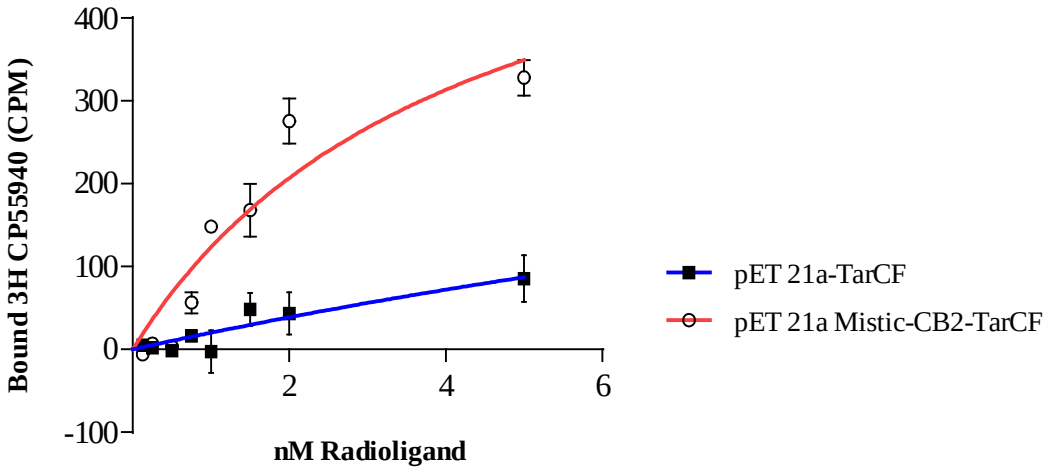

Figure 4.9. Comparative difference of saturation binding assay of membrane fractions.

Membrane fractions were prepared from *E. coli* C43(DE3) cells transformed with pET21a-Mistic-CB2-TarCF and pET 21a-TarCF (negative control). Specific Binding was obtained as a difference between the Total and Non-Specific binding. Graph above shows the difference of Specific Binding between the fusion construct and the negative control.

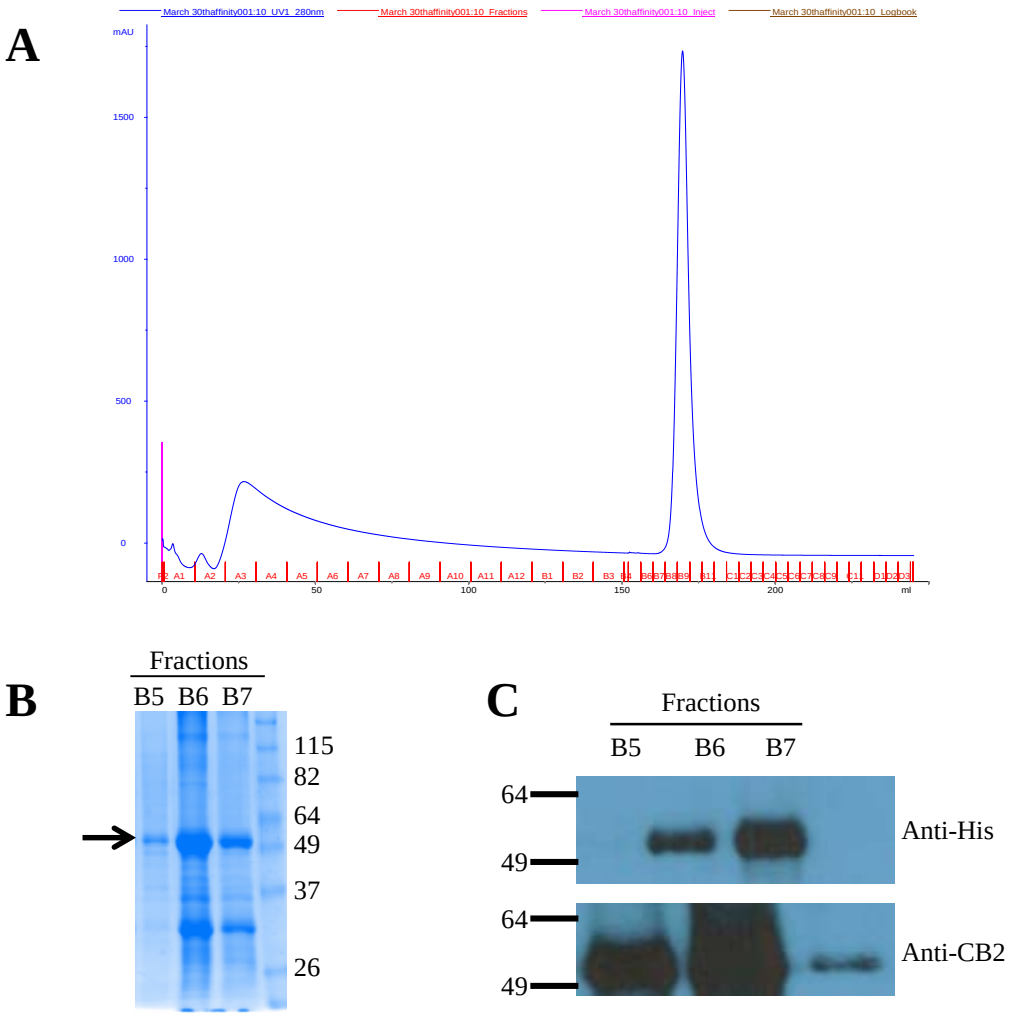

Figure 4.10. pH gradient IMAC purification chromatogram of the TrpΔLE-Xa-CB2 fusion construct.

(A) TrpΔLE-Xa-CB2 (53kDa) was solubilized from enriched inclusion bodies using 1% SDS and was loaded into a pre-equilibrated IMAC column at pH-8.0. Non specific and Specific proteins were eluted at pH -7.0 and 6.0 respectively. Subsequent SDS-PAGE of eluted fractions and its CBB staining is shown in **(B)** and were also verified by **(C)** Western Blotting with Anti-His and Anti -CB2 antibodies.

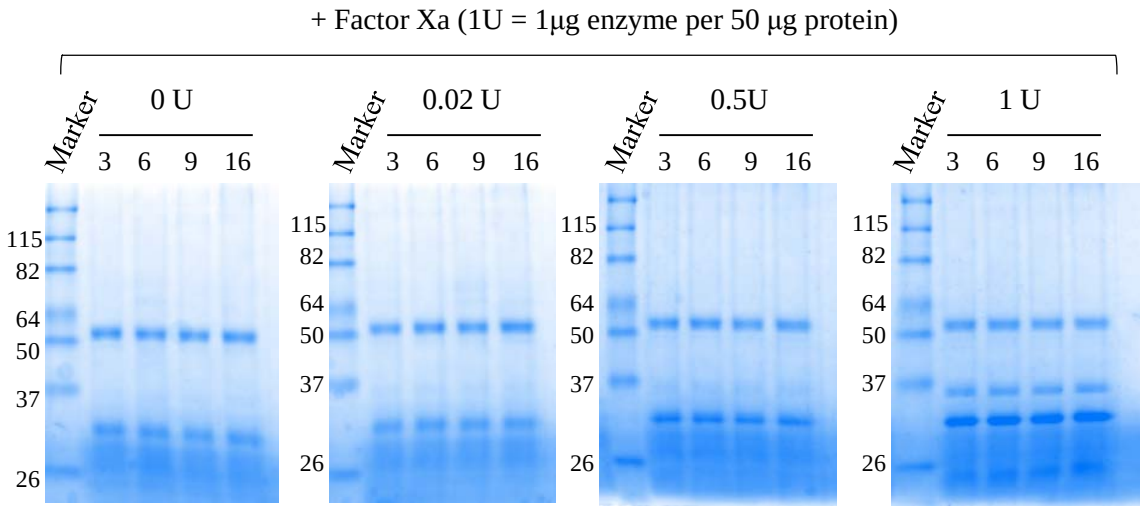

Figure 4.11. Optimization of TrpΔLE-Xa-CB2 cleavage by Factor Xa.

Process optimization was implemented to improve the efficiency of Factor Xa cleavage. The exchanged TrpΔLE-Xa-CB2 protein was incubated with different concentrations (0,0.02,0.5 and 1µg) of Factor Xa per 50 µg of TrpΔLE fused CB2 receptor for 3,6,9,16 (overnight) hours (as indicated over lanes corresponding lanes).

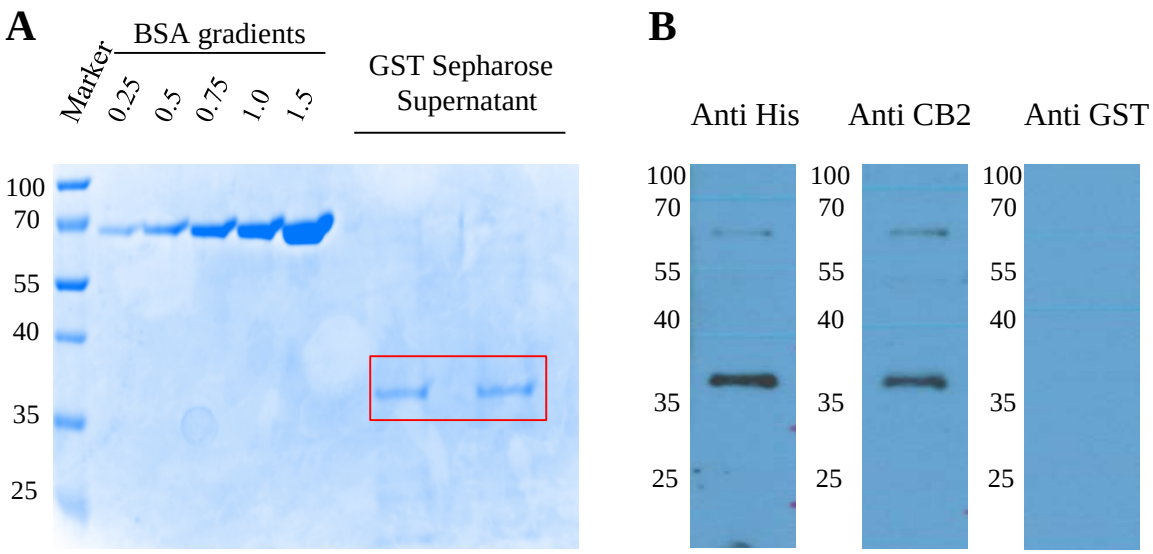

Figure 4.12. Clarification of the cleaved GST-CB2 protein.

(A) To clarify the eluent from the uncleaved parent and the cleaved released GST the mixture was incubated with GST Sepharose resin pre-equilibrated with the cleavage buffer. Resin was separated from the liquid phase and the supernatant was separated on a 10% SDS PAGE and CBB stained. **(B)** Supernatant was probed with Anti-His, Anti-CB2 and Anti-GST antibodies.

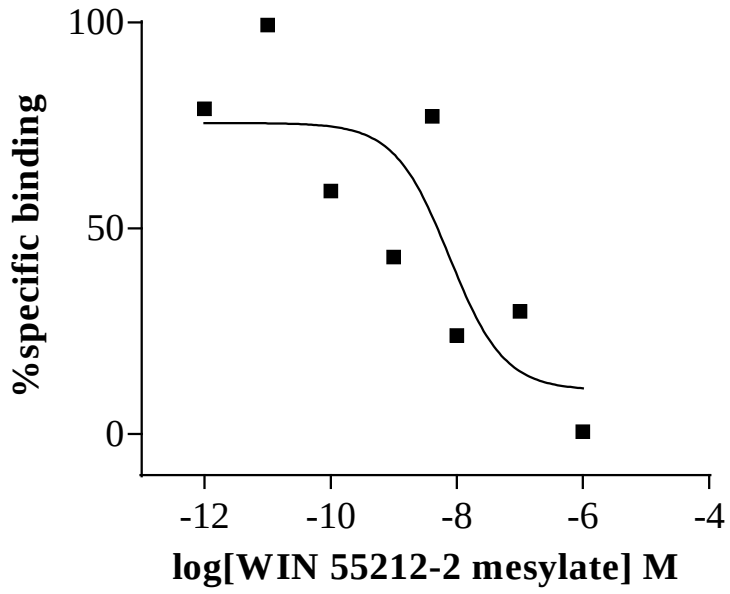

Figure 4.13. Competitive binding of CB2 in DMPC to WIN 55212-2 mesylate

Competitive displacement of the ^{3}H-CP55,940 was obtained by using an increased amount of cold ligands. Binding profile of the agonist, WIN 55212-2 mesylate salt form by displacing the ^{3}H CP55,940.

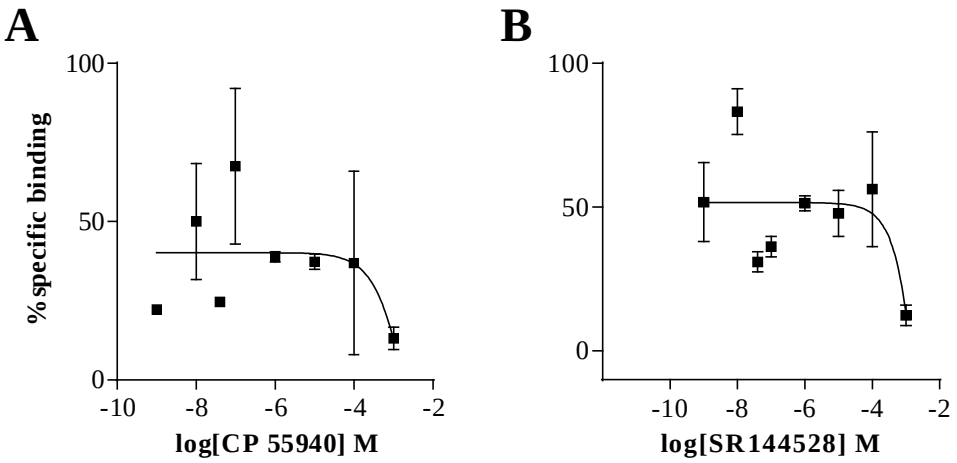

Figure 4.14. Competitive displacement of the ^{3}H-CP 55, 940 with the CB2 refolded in Amphipol A8-35

CB2 receptor refolded in Amphipols was tested for ligand displacement capability with **(A)** cold CP 55,940 and **(B)** inverse agonist SR 144528.

APPENDIX B

SUPPLEMENTARY INFORMATION

B.1 FOOTNOTES ON CHAPTER 2

B.1.1 Expression of the CB2 receptor with fusion partners

We used the bacterial thioredoxin TrxA tag as a C terminal fusion partner for the expression of CB2 receptor tagged to Mistic in the N-terminal to generate the final construct Mistic-CB2-TrxA. The thioredoxin tag has been applied in several protein production applications as it increases the solubility of the overall fusion construct. A comparable amount of Mistic-CB2-TarCFand the Mistic-CB2-TrxA was observed by Western Blotting (Figure 4.15). However the construct with the Mistic and the TarCF was used for further expression purification studies due to the novelty of the fusion partners. This demonstrates that the Mistic is a versatile N terminal fusion partner and the Mistic and TrxA in combination may also be used for GPCR membrane targeted expression and prurification.

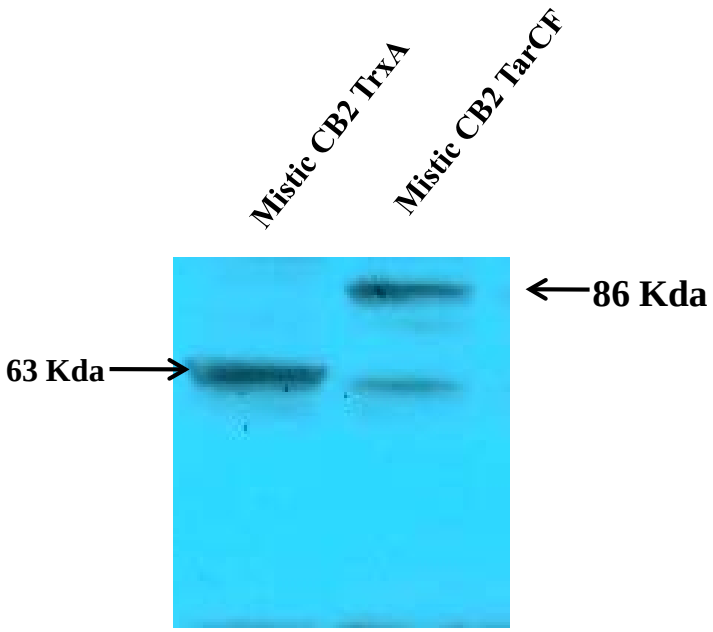

Figure 4.15. Comparison of fusion protein expression levels.

Western Blot analysis of whole cell lysates from bacteria transformed with the constructs Mistic-CB2-TrxA (63 kDa) and Mistic-CB2-TarCF (86 kDa).

B.1.2 Comparison of the overall RMSD of the CB2 receptor between receptor homology models with or without the fusion partners

To compare the differences between CB2 structure and disposition with or without the fusion partners we compared the root mean square deviation (RMSD) between the CB2 with or without fusion partners. The CB2 homology model (14517981) (which will be used for comparison with CB2 was fused with the Mistic and the TarCF via linker) was connected to the Mistic and the TarCF using linker segments. The NMR structure of Mistic (PDB:1YGM) was obtained from

PDB protein data bank and the homology model of the TarCF was obtained from the crystal of the serine chemoreceptor Tsr (PDB:2D4U). The structure of the fusion protein was arrived at by using the orchestar function from the Sybyl 8.0. The structures of the Mistic, CB2 and TarCF were incorporated without any further modification. The linker regions were generated by homology modeling and using proteins fragments which have very high sequence similarity and for which the structure are known. All components were connected by peptide bond (peptide bond linkage *in-silico*) and the overall fusion protein was subjected to energy minimization. The fusion protein model was saved and imported back to Sybyl window with CB2 alone. Overall RMSD value was 2.021 and structural deviations were seen maximally in the intracellular loop IL3 region (Figure 4.16). This result suggested before our functional activity assays, that Mistic and TarCF when linked with the linker peptides to the CB2 should not lead to significant structural loss of the receptor.

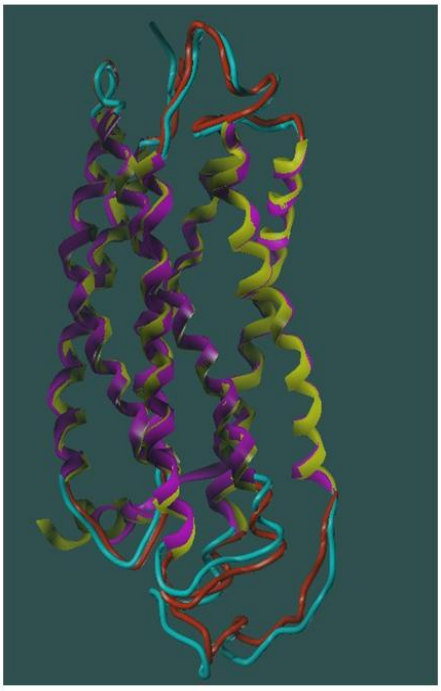

Figure 4.16. Comparison of the backbone RMSD of the CB2 receptor virtual model and fusion protein.

Tripos Sybyl software 8.0 was used for fusion protein model building and RMSD measurement of CB2 receptor virtual model (Xie *et. al.* 2003) (purple and blue) with the CB2 fusion protein Mistic-CB2-TarCF (yellow and red). Overall RMSD between the models is 2.021 and structural deviations were seen maximally in the intracellular loop IL3 region.

B.2 FOOTNOTES ON CHAPTER 3

TROUBLESHOOTING STEPS

B.2.1 Purification of the GST CB2 fusion protein inclusion bodies- process complications and troubleshooting steps

Presence of excess amount **of DNA**

Processing of the cell pellets to arrive at the enriched inclusion bodies and preparation of the load material from the enriched inclusion bodies both have the problem of the presence of excess amounts released DNA. The excess DNA will lead to the inefficient separation of the IBs from the solubilized protein during the IB enrichment steps. This is due to the formation of sloppy like material which prevent the separation of the soluble and insoluble materials. .Further the presence of DNA even in moderate amounts leads to high resistance during the filteration step prior to loading the sample to the affinity chromatography column. Loading unfiltered material might block the column or tubings in the AKTA system and hence it is extremely important to have the load material filtered before loading to the affinity column.

To remove the excess amounts of DNA during the IB enrichment steps the sample must be well sonicated on ice. This will allow breakdown of majority of the contaminant DNA by shearing force. Care must be taken though that the sample is always maintained on ice and heating does not result as a result of sonication. Following sonication small amounts of universal nuclease might be added to the slurry to lead to further chemical breakdown. On the other hand, removing DNA from unclear load material should be done mostly by chemical lysis as the enriched GST

CB2 in the load material is much more susceptible for breakdown during harsh sonication. A couple of short burst (2-5 secs) of sonications should be done on ice however the majority of the DNA removal should be carried out by chemical lysis.

Removal of Hydrophobic contaminants

The process of IB enrichment can be monitored by noticing the color of the pellet. The enriched IB pellet should be whitish in color. If the pellet material is brownish/blackish in color it is suggestive of the presence of hydrophobic contaminants. Hydrophobic proteins can be removed by washing the pellet repeatedly with base buffer (20 mM Tris HCl, 250 mM NaCl, pH-7.5) supplemented with 0.5% Triton X-100. However care should be taken to achieve a balance between removal of unwanted material and loss of the precious enriched IB. Resuspending the enriched IB in the base buffer should result in a “milk-like” appearance.

Purification of the solubilized IBs

The solubilized IBs are stabilized within 1% Lauryl Sarkosyl and are maintained within reduced conditions with 10 mM β-Mercaptoethanol. The presence of the beta –ME leads to the color change of the IMAC column. We have observed no differences in the binding efficiency of the protein of interest (judged by the final protein yield). However, it would be important to wash the column with >20CV of the thrombin cleavage buffer prior to injecting the thrombin protease. This not only ensures the complete exchange of Sarkosyl with DDM but also leads to the complete removal of the reducing agent.

On column cleavage

To ensure better thrombin cleavage the following should be very useful. The thrombin protease should be resuspended from powder using the thrombin cleavage freshly before injection to the column. The buffer containing the protease (1CV) should be injected into the column with a relatively higher flow rate to ensure that the protease spreads up within the column.

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