Purification and Characterization of Heterologously Produced Cannabinoid Receptor 1 and G proteins

Dissertation

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Datum der Disputation:

To my mother..

Diese Doktorarbeit wurde vom 12. September 2002 bis zum 30. April 2007 unter Leitung von Prof. Dr. Hartmut Michel und Dr. Christoph Reinhart in der Abteilung für Molekulare Membranbiologie am Max-Planck Institute für Biophysik in Frankfurt am Main durchgeführt.

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine weiteren Hilfsmitten und Quellen als die hier aufgeführten verwendet habe.

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Abbreviations:

2D	Two dimensional
3D	Three dimensional
AC	Adenylyl Cyclase
AM251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
AM630	[(6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl)(4-methoxyphenyl)methanone]
AP	Alkaline phosphatase
APS	Ammonium persulphate
βMe	β-Mercaptoethanol
BCA	Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolylphosphate, 4-toluidine salt
bp	Base pairs
BSA	Bovine serum albumin
$C_{12}E_8$	Dodecyloctaethylene glycol ether
cAMP	3',5'-cyclic-adenosine monophosphate
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
cDNA	complementary deoxyribonucleic acid
CFP	Cyan fluorescent protein
CHAPS	3-[N-(3-Cholamidopropyl)-dimethyl-ammonio]-1-propansulfonate
CHS	Cholesterol hemisuccinate
CMC	Critical Micelle Concentration
CNS	Central nervous system
cpm	counts per minute
Cymal 6	5-Cyclohexyl-1-pentyl-β-D-maltoside
Da	Dalton
DM	n-Dodecyl-β-D-maltoside
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

dpm	disintegrations per minute
DTT	1,4 Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FCS	Foetal calf serum
Fos12	n-dodecylphoscholine
GDP	Guanosine 5'-diphosphate
Gα	α subunit of heterotrimeric G-protein
Gβ	β subunit of heterotrimeric G-protein
Gγ	γ subunit of heterotrimeric G-protein
GTP	Guanosine triphosphate
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
GppNp	5'-Guanylylimidodi-phosphate
g	Centrifugal force
GPCR	G protein coupled receptor
G protein	Guanine nucleotide binding protein
hr	hour(s)
Hepes	N-(2-Hydroxyethyl)-Piperazine-N'-(2-ethylsulphonic acid)
HU210	(6aR)-trans-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol
IMAC	Immobilized metal affinity chromatography
Kb	Kilo basepairs
K_D	Dissociation constant
KDa	Kilo Daltons
Kan	Kanamycin
LB	Luria-Bertani medium
LDAO	N,N-dimethyldodecylamine-N-oxide
LM	n-Dodecyl-β-D-maltoside
min	minute(s)
MOI	Multiplicity of infection
NA	not applicable
NTA	Nitrilo triacetic acid
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylenimine
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene fluoride
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
TEMED	N,N',N',N'-Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
UC	Ultracentrifuge
v/v	Volume per volume
w/v	Weight per volume
WIN 55,212-2	(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethly)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YFP	Yellow fluorescent protein
YNB	Yeast Nitrogen Base

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Zusammenfassung:

G-Protein gekoppelte Rezeptoren (GPCRs) bilden die größte Hauptklasse innerhalb jener Gruppe von Transmembranproteinen, die ein extrazelluläres Signal in eine spezifische intrazelluläre Reaktion (Signaltransduktion) umwandeln. Etwa 3% des humanen Genoms codieren für GPCRs, wobei diese wiederum den Angriffspunkt für 40-50% der zurzeit auf dem Markt befindlichen Pharmaka bilden. Diese Angaben unterstreichen die Wichtigkeit der GPCR-Superfamilie und verdeutlichen die Notwendigkeit für ein tief greifendes Verständnis ihrer Funktionsweise. Die externen Stimuli, die über GPCRs eine spezifische intrazelluläre Reaktion auslösen können, sind sehr vielfältig. Sie reichen von Licht, Geruchs- und Geschmacksstoffen über Amine, Peptide, Lipiden und Nukleotiden bis hin zu Ionen wie etwa Ca^{2+}. Die ligandeninduzierte Konformationsänderung des Rezeptors überträgt das Signal auf ein cytosolisches Guanin-Nukleotide bindendes Protein (G-Protein), das daraufhin seinerseits eine Kaskade zellulärer Reaktionen startet.

Während in den letzten Jahrzehnten vielfältigste biochemische und pharmakologische Daten über diese Proteinfamilie gesammelt werden konnten, sind die vorhandenen Strukturinformationen immer noch sehr ungenügend. Die einzige für diese Proteinsuperfamilie bisher verfügbare dreidimensionale Struktur hoher Auflösung ist die des bovinen Rhodopsins. Einer der Hauptgründe für die Schwierigkeit der Stukturaufklärung bei GPCRs ist die mangelnde Verfügbarkeit des Zielproteins selbst: In ihren nativen Geweben werden GPCRs üblicherweise nur in verschwindend geringen Mengen exprimiert. Daher ist die Reinigung der für Strukturuntersuchungen benötigten Mengen aus nativen Geweben stets sehr zeitaufwendig und in vielen Fällen gar nicht möglich. Um diese erste Hürde auf dem Weg der Strukturaufklärung zu überwinden, wurde eine Vielzahl heterologer Expressionssysteme etabliert. Eine weitere Schwierigkeit bei der Strukturbestimmung von GPCRs liegt in der Tatsache begründet, dass es sich bei dieser Proteinfamilie um integrale Membranproteine handelt, und bei diesen eine Strukturbestimmung generell eine große Herausforderung darstellt. Während zurzeit bei den löslichen Proteinen bereits mehr als 13000 hochauflösende Strukturen zur Verfügung

stehen, sind es bei den Membranproteinen gerade einmal etwa 120. Einer der Gründe für dieses dramatische Ungleichgewicht dürften die nur recht kleinen hydrophilen Oberflächenbereiche der Membranproteine sein, da hierdurch die Möglichkeiten für Kristallkontakte zwischen den einzelnen Proteinmolekülen einschränkt wird. Bei GPCRs letztlich werden die einzigen hydrophilen Bereiche von den die Transmembranhelices verbindenden intra- und extrazellulären Schleifen sowie den N- und C-Termini gebildet. Die genannten Bereiche sind sowohl relativ klein als auch strukturell eher flexibel, was eine Kristallisation weiter erschwert.

Die vorliegende Arbeit ist ein Versuch, Wege zur Lösung der oben genannten Probleme aufzuzeigen. Ziel des Projektes war die Verwendung von G-Proteinen, um zusammen mit einem GPCR einen Komplex zu schaffen, dessen hydrophiele Beireiche stark vergrößert sind. Es handelt sich hierbei um einen der Protein-Kokristallisation mit Fv-Antikörperfragmenten entsprechenden Ansatz. Da es sich bei G-Proteinen zudem um den physiologischen Bindungs- und Interaktionspartner der GPCRs handelt, wäre die Struktur eines solchen Komplexes zudem von besonderem Interesse. Bei G-Proteinen handelt es sich um heterotrimere Proteine, mit je einer α-, β- und γ-Untereinheit, wobei der α-Untereinheit (G_α) bei der Wechselwirkung mit dem GPCR die größte Bedeutung zukommt. Im humanen Genom wurden 21 verschiedenartige α-Untereinheiten identifiziert, wobei es sich jedoch bei einigen von ihnen um Splice-Varianten handelt. In der vorliegenden Arbeit wurden insgesamt 16 verschiedene G_α-Untereinheiten unter Verwendung des GATEWAY®-Systems in den Vektor pDEST14 kloniert und in *Escherichia coli* expremiert. Zur Bestimmung der optimalen Induktionsbedingungen und -zeiten kam ein Hochdurchsatz-Screen auf *Dotblot*-Basis zum Einsatz. Bei fünf der in *E. coli* exprimierten G_α-Untereinheiten war es möglich, diese mittels einer Kombination aus Immobilisierter Metallchelat-Affinitätschromatographie (IMAC) und Ionenaustausch-Chromatographie rein darzustellen. Die Bemühungen, auch die G_β- und G_γ-Untereinheiten in *E. coli* herzustellen, waren hingegen nicht erfolgreich. $G_{\alpha q}$, das zu jenen G_α-Untereinheiten zählt, die sich nicht in *E. coli* expremieren ließen, wurde erfolgreich in der methylotrophen Hefe *Pichia Pastoris* produziert. Das aus *P. pastoris* gereinigte $G_{\alpha q}$-Protein konnte für Kristallisationsansätze genutzt werden. Unter einer Bedingung wurden Kristalle erhalten, die eine Röntgenbeugung bis 6,5 Å zeigten. Zurzeit besteht ein Problem bei der Produktion dieser Untereinheit, das noch der Klärung bedarf. Die G_α-Untereinheiten, die rein

dargestellt werden konnten, wurden hinsichtlich ihrer Aktivität in Detergenz analysiert. Hierbei zeigte sich, dass die Mitglieder der $G_{\alpha s}$-Subklasse (stimulieren die Adenylat-Cyclase) in Detergenz keinerlei Aktivität aufwiesen. Die Mitglieder der $G_{\alpha i}$-Subklasse (inhibieren die Adenylat-Cyclase) behielten hingegen in den meisten der getesteten Detergenzien ihre Fähigkeit der Guanosintriphosphat(GTP)-Bindung bei. Aus diesen Ergebnissen folgt, dass die Kokristallisation eines GPCRs mit $G_{\alpha s}$ wohl nicht sinnvoll ist, da bei solchen Ansätzen aufgrund des Rezeptors stets Detergenz anwesend sein muss. Zu diesem Zeitpunkt des Projektes standen in unserem Institut nur wenige an $G_{\alpha i}$ koppelnde GPCRs zur Verfügung, die in ausreichenden Mengen rein dargestellt werden konnten um die $G_{\alpha i}$-Bindung zu studieren. Des Weiteren legten andere Veröffentlichungen nahe, dass in *E. coli* produzierte G_{α}-Untereinheiten alleine nicht in der Lage sind, an GPCRs zu binden. Aus diesen Gründen wurde das Projekt dahingehend erweitert, einen jener GPCRs zu gewinnen, von denen berichtet wurde, sie lägen in der Zelle bereits ohne gebundenen Liganden in einem Komplex mit ihrem G-Protein vor. Von der Reinigung eines solchen physiologischen Komplexes wurden sich erhebliche Vorteile für die Kristallisation versprochen.

Für die heterologe Produktion und anschließende Reinigung unter oben genanntem Aspekt wurden die Cannabinoid-Rezeptoren ausgewählt. Zurzeit unterscheidet man zwei Subtypen von Cannabinoid-Rezeptoren. Zum einen den Cannabinoid-Rezeptor 1, der vornehmlich im zentralen und peripheren Nervensystem vorkommt und zum anderen den Cannabinoid-Rezeptor 2, welcher in Immunzellen gefunden wird. Beide Subtypen koppeln an $G_{\alpha i/o}$. Aus ihrer histologischen Verteilung wurde gefolgert, dass der Cannabinoid-Rezeptor 1 vermutlich eine neuroprotektive Funktion hat, während der Cannabinoid-Rezeptor 2 immunosuppressiv wirkt. Die Cannabinoid-Rezeptoren sind zudem der Angriffspunkt der Inhaltsstoffe von Cannabis (Marihuana, Haschisch), der am weitesten verbreiteten Rauschmittel. Die Verwendung von Marihuana geht jedoch über die eines bloßen Rauschmittels hinaus, da es bereits seit 2000 vor Christus zur Behandlung einer Reihe von Krankheiten eingesetzt wird. Der Pflanzenextrakt aus *Cannabis sativa* war z.B. dafür bekannt, Schmerzen zu lindern, Übelkeit zu unterdrücken und den Appetit zu fördern. Nach wissenschaftlicher Analyse des *Cannabis sativa*-Extraktes konnte Δ9-Tetrahydro-cannabinol als hauptsächlicher aktiver Bestandteil identifiziert werden. Der Pflanzenextrakt enthält jedoch etwa 50 weitere, diesem verwandte Verbindungen, die mit unterschiedlichen

Affinitäten an die Cannabinoid-Rezeptoren binden und eventuell eigene pharmakologische Wirkungen entfalten. Weitreichende Forschungen während der letzten Jahrzehnte haben demonstriert, dass es sich bei den Cannabinoid-Rezeptoren um viel versprechende Zielproteine bei der Bekämpfung einer Vielzahl von Krankheitssymptomen handelt.

Die Produktion des Cannabinoid-Rezeptors 2 wurde zunächst im *Pichia pastoris* Expressionssystem untersucht. Das Expressionskonstrukt beinhaltete ein N-terminales Dekahistidin-Anhängsel sowie C-terminal die Biotinylierungsdomäne der Transcarboxylase aus *Propionibacterium shermanii* (Biotag). Leider stellte sich das produzierte Protein als ausgesprochen heterogen heraus, in den Zellmembranen waren mehrere oligomere Formen vorhanden, sowie verschiedene Degradationsprodukte. Versuche zur Reinigung des Proteins erwiesen sich sowohl hinsichtlich der erreichten Reinheit als auch der Ausbeute als ungenügend. Zudem zeigte die analytische Gelfiltrations-Chromatographie, dass der Großteil des Proteins aggregiert war.

Als alternatives Expressionssystem wurde daher die Bakulovirus-vermittelte Expression in Insektenzellen untersucht. Hierbei lag der Fokus mehr auf der heterologen Produktion des Cannabinoid-Rezeptors 1, da bei diesem zum einen detaillierteres Verständnis der biochemischen Vorgänge vorliegt und er zum anderen die größere pharmakologische Wichtigkeit besitzt. Für die heterologe Produktion wurde sowohl eine Vollängenversion des Rezeptors als auch eine Version mit deletiertem C-Terminus verwendet. Zur Reinigung mittels Affinitätschromatographie wurden von beiden Versionen Konstrukte erstellt, die mit einem N-terminalen Polyhistidin-Anhängsel versehen waren und C-terminal entweder das Strep II-Anhängsel oder das Biotag trugen. Bei sämtlichen getesteten Konstrukten war eine Überproduktion in *Spodoptera frugiperda (Sf9)* Zellen zu beobachten.

Mit N-terminalem Decahistidin-Anhängsel und C-terminalem Strep II-Anhängsel betrug das Produktionsniveau (B_{max}) für das Vollängenkonstrukt 40 pmol/mg und 53 pmol/mg für die verkürzte Version. Diese Mengen sind gut doppelt so hoch wie die besten bis jetzt veröffentlichten Angaben und bilden eine gute Grundlage für eine nachfolgende Reinigung des Rezeptors. Die Charakterisierung des Rezeptors mittels Radioligandenbindung zeigte, dass die Agonistenbindung des Cannabinoid-Rezeptors 1 von

der Anwesenheit von Magnesiumionen abhängig war, während die Antagonistenbinung Mg^{2+}-unabhängig erfolgte. Ferner führten hohe Natriumchlorid-Konzentrationen im Reaktionspuffer zu einer verminderten Agonisten-Bindung, während sie die Antagonisten-Bindung nicht beeinflussten. Im Gegensatz zu anderen GPCRs konnte für den Cannabinoid-Rezeptors 1 auch dann noch Ligandenbindung gemessen werden, wenn 1,4-Dithiothreit (DTT) in höheren Konzentrationen (10 mM) anwesend war. Andererseits führte die Mehrzahl der getesteten Detergenzien zu einer Verringerung der Ligandenbindung. Aufgrund hoher unspezifischer Bindung des Radioliganden war ein Nachweis der Ligandenbindung für den solubilisierten sowie für den gereinigten Rezeptor bisher nicht erfolgreich.

Wie den bereits vorliegenden Publikationen anderer Gruppen zu entnehmen war, stellte die reine Darstellung dieses Rezeptors eine erhebliche Herausforderung dar. Die nach Reinigung mittels IMAC vorliegende Präparation wies dann auch eine Reinheit von bestenfalls 50% auf. Eine nachfolgende zweite Affinitätschromatographie unter Verwendung von monomerer Avidin-Matrix (Biotag) oder *Strep*-Tactin-Agarose (Strep II-Anhängsel) führte zu einer drastischen Verringerung der Ausbeute. Im Falle der *Strep*-Tactin-Agarose erfolgte die Bindung des rekombinanten Rezeptors mit nur geringer Effizienz, während die Bindung an die monomere Avidin-Matrix überwiegend irreversibel war. Auch nach der zweiten Affinitätschromatographie wies die Präparation noch verschiedene Verunreinigungen auf und Variationen der Waschbedingungen konnten keine Verbesserung herbeiführen. Daraufhin wurde die Reinigung des Rezeptors mittels verschiedener IMAC-Matrices erprobt. Unter Verwendung einer von der Firma Sigma vertriebenen Ni-NTA-Matrix mit der Bezeichnung His-Select konnte ein deutlich verbessertes Reinigungsprofil erhalten werden. Unter optimierten Bedingungen wurde zwar eine Reinheit von etwa 80% erreicht, die Ausbeute lag hierbei allerdings lediglich bei 20% und war damit für den Beginn von Strukturuntersuchungen nicht genügend. Das Gelfiltrationsprofil des gereinigten Rezeptors war zudem inhomogen und deutete auf verschiedene oligomere Formen in der Präparation hin. Hier sind noch weitere Bemühungen erforderlich, um sowohl die Ausbeute als auch die Reinheit/Homogenität der Rezeptor-Präparationen so weit zu steigern, dass Kristallisationsversuche unternommen werden können.

Von den Cannabinoid-Rezeptoren ist bekannt, dass sie in der Zelle auch ohne gebundenen Liganden bereits in einem Komplex mit dem G-Protein vorliegen (GPCR/G-Protein-Komplex). Diese Eigenschaft könnte für die Kristallisation durchaus von großem Nutzen sein. Der GPCR/G-Protein-Komplex existiert sowohl in einer aktiven als auch einer inaktiven Form. Während der aktive GPCR/G-Protein-Komplex die konstitutiv aktive Form des Rezeptors darstellt, sind beide Formen an der G-Protein Sequestrierung in der Zelle beteiligt. Die Existenz beider genanter GPCR/G-Protein-Komplex wurde in dieser Arbeit mittels Fluoreszenztechniken untersucht. Durch Fluoreszenz-Resonanz-Energietransfer (FRET)-Messungen konnte gezeigt werden, dass der Cannabinoid-Rezeptor 1 in dem beschriebenen Komplex mit G_{i1} vorliegt. Für den C-terminal verkürzten Cannabinoid-Rezeptor 1 in Fusion mit dem gelb fluoreszierenden Protein (CB1-417-YFP) und G_{i1} in Fusion mit dem cyan fluoreszierenden Protein (G_{il}-CFP) konnte die Kolokalisation innerhalb der Zelle auch bei Abwesenheit eines Liganden nachgewiesen werden. Mit einem Guanosintriphosphat-Bindungsassay an Zellmembranen konnte ferner nachgewiesen werden, dass der aktive GPCR/G-Protein-Komplex auch in der Abwesenheit eines Agonisten vorliegt. In einem weiteren Experiment wurden die Membranen von Zellen, die das verkürzte Rezeptorkonstrukt mit N-terminalem Flag-Anhängsel und das heterotrimere G-Protein koexprimierten solubilisiert und erfolgreich eine Koimmunopräzipitation des Rezeptor/G-Protein-Komplexes mittels Anti-Flag M2-Agarose durchgeführt.

Die in der vorliegenden Arbeit erhaltenen Ergebnisse zeigen, dass die C-terminal verkürzte Form des Cannabinoid-Rezeptors 1 in Insektenzellen funktionell produziert werden konnte, wobei die erreichten Produktionsniveaus weit über denen in bisherigen Veröffentlichungen liegen. Ferner konnte durch FRET-Experimente gezeigt werden, dass dieser Rezeptor auch in Abwesenheit eines Liganden in einem Komplex mit dem G-Protein vorliegt. Diese Ergebnisse wurden durch die erfolgreiche Koimmunopräzipitaion des Rezeptor/G-Protein-Komplexes bestätigt. Weiterführende Untersuchungen sind erforderlich, um zu überprüfen, in wie weit dieser Rezeptor/G-Protein-Komplexe gereinigt und eventuell für die Kokristallisation eingesetzt werden kann.

Summary:

G protein coupled receptors form the largest group of transmembrane proteins, which are involved in signal transduction and are targeted directly or indirectly by 40-50% of the drugs in the market. Even though a lot of biochemical and pharmacological information was acquired for these receptors in the past decades, structural information is still insufficient. G protein coupled receptors are expressed in a very minute scale in the tissues. Purification of G protein coupled receptors, in amounts needed for structural studies, from native tissue is tedious and almost impossible. To overcome this first hurdle of insufficient protein, several heterologous protein expression systems are being used. Another difficulty in structural determination of a G protein coupled receptor is that it is a membrane protein. Membrane proteins are difficult targets for structural studies. One of the possible reasons is the little hydrophilic surface area on the membrane protein, reducing the chances of crystal contact between the molecules.

The present work is an attempt to investigate possible ways to overcome these problems. Aim of the project was to use G proteins to increase the hydrophilic area of the G protein coupled receptor. G protein is a physiological partner to the G protein coupled receptor which makes the complex functionally relevant. In the present work five Gα proteins were purified to homogeneity by a two step purification using metal affinity and ion-exchange chromatography. The Gα subunits purified were tested for their detergent susceptibility. It was found that only some G proteins were active in the presence of detergent. Observation from contemporary reports also suggest that the Gα proteins expressed in *Escherichia coli*, alone may not be sufficient to bind to the G protein coupled receptors in solution. So the project was extended towards expressing a G protein coupled receptor which was reported to exist in a complex with the G proteins, in the cells. Purifying such a functional complex could be more beneficial to use for crystallization. Cannabinoid receptors were chosen for heterologous expression and purification. Production of recombinant cannabinoid receptor 2 was investigated in *Pichia pastoris*. The protein obtained was highly heterogenous. There were several oligomeric forms as well as

degradation products in the cell membranes. Most of the protein was lost in the purification steps leading to a poor yield. Several oligomeric forms and other impurities were still present in the protein sample after purification. Alternatively, a baculovirus mediated insect cell expression system was investigated, to produce the receptors. Cannabinoid receptor 1 was investigated in insect cell expression system because of its better biochemical understanding and pharmacological importance than cannabinoid receptor 2. Cannabinoid receptor 1 was produced in two forms, a full length and a distal carboxy terminal truncated version. All the several gene constructs made could be expressed in the *Spodoptera frugiperda (Sf9)* insect cells. Expression levels (Bmax) for the constructs with a deca-histidine tag at the amino terminus and Strep-tagII at the carboxy terminus were 40 pmol/mg and 53 pmol/mg respectively, for full length and truncated versions. These expression levels are 2 fold higher than the levels reported till now in the literature. As was quite evident from previous experiences of other research groups, purification of this receptor was a challenge. Protein purified from immobilized metal affinity chromatography (Ni-nitrilo tri acetate)(Ni-NTA) was not even 50% pure. A second purification by immobilized monomeric avidin or Streptactin agarose, making use of Biotag and Strep-tagII respectively, drastically reduced the protein recovery. Later on, purification of receptor was investigated on different metal chelating resins. His-Select, a Ni-NTA based matrix from Sigma, with much lesser density than Ni-NTA from Qiagen, showed a better purification profile. Purification was optimized to get 80% homogeneity but with low yield (20%). Further efforts are needed to improve the yield and purity of the receptor, to use it for crystallization.

Cannabinoid receptors are known to exist in a precoupled form to G proteins in the cells. The existence of such precoupled forms of the receptor was investigated using the fluorescence techniques. Guanosine-5-triphosphate binding assay on the cell membranes, in the absence of agonists confirmed the active precoupled form of the receptor. It was found that it is possible to co-immunoprecipitate the complex. These results show that the truncated cannabinoid receptor can be produced in functional form in insect cells in much higher yields than reported. This receptor exists as a complex with G proteins even in the absence of ligands. It was also shown that the receptor/G protein complex can be co-immunoprecipitated. Further work is required to investigate the possibility of purifying this complex to use it for co-crystallization.

1. Introduction..

1 INTRODUCTION

1.1 Membrane proteins

Cells are the smallest functional units of all living organisms. These cells exist alone or as a unit of a multi-cellular organism. Cells, co-ordinate the functions of all basic reactions within itself, as well as they respond in time to the external environment. A continuous plasma membrane separates the inside and outside of the cell. Eukaryotic cells have organelles, which perform specific functions and need specific environments within them. These organelles are also surrounded by the lipid membranes. These lipid membranes are impermeable for hydrophilic molecules. But the cell has to take up many hydrophilic components, e.g. nutrients, to survive. To allow transport across the membrane, many proteins are embedded in the cell membranes. These proteins possess the function of transporting the essential chemical components into or out of the cell. Cells need to pass on a lot of information from the outside to the inside to co-ordinate the functions and they also have to communicate with the surrounding cells. How do they communicate? Again, proteins embedded in the membrane carry on the function.

The plasma membrane is a bilayer of phospholipids. The outer and inner surfaces of the lipid bilayer are hydrophilic, which are in contact with the universal solvent, water and its solutes. The inside of the double layer made from the fatty acyl tails, is hydrophobic or lipophilic which renders the bilayer impermeable for the hydrophilic molecules. Proteins embedded in the membranes render them permeable and connect the exterior and interior of the cell. Proteins in the membrane are thus called membrane proteins. The transmembrane parts of the membrane proteins are generally hydrophobic in nature, so that they can stay within the lipophobic bilayer.

Proteins which are lying on the surface of the lipid bilayer are called peripheral membrane proteins or extrinsic membrane proteins. Proteins which span the bilayer or deeply incorporated in the lipid bilayer are called intrinsic membrane proteins.

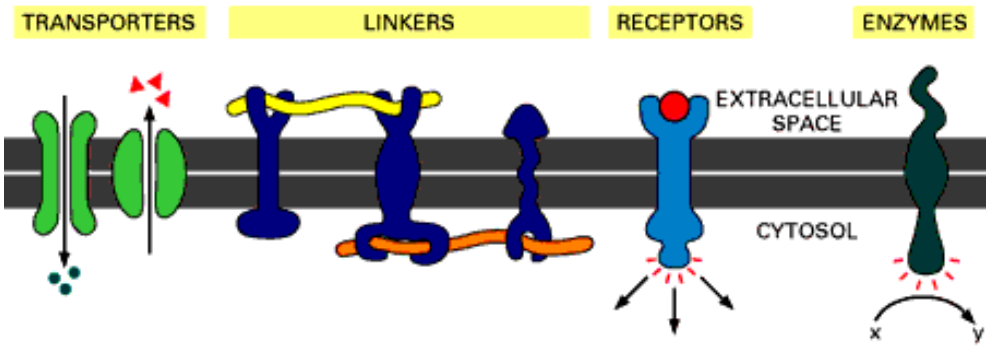


Fig 1.1: Representation of a few types of membrane proteins.

As represented in Fig 1.1, there are several functions associated with the membrane proteins. Transport of ions and other biomolecules, linkers (integrin) forming a part of cytoskeleton, signal transduction by receptors (e.g. GPCRs), synthesis or degradation of certain biomolecules by enzymes (cAMP synthase, ATP synthase) are few of many functions. Proteins involved in transporting the chemical components are generalized as membrane transporters. These proteins are named pumps or channels according to their mechanism of action. Passive transport is a mode where no energy is utilized to permeate the ions or chemical components across the channels. Diffusion is a simple way of passive transport. Selective transport of molecules across the membrane through the channels either non-gated or gated also does not utilize any energy. In active transport of molecules energy is needed in one form or another. Primary active transporters like P-type ATPases utilize the energy stored in ATP molecules to transport the ions across the membrane. These are generally termed as pumps. Secondary active transporters utilize energy differences because of the coupled transport to other molecules. It can be a symport where both molecules move in the same direction or antiport where the molecules move in opposite directions.

1.2 Cell surface receptors

The concept of receptors to describe the interaction of drugs with cells is believed to be put forward by Paul Ehrlich(1854-1915) and John Newport Langley(1854-1936), which was later mathematically modeled by Alfred J. Clarke (1885-1941), considered as father of modern receptor theory (Kenakin *et al*., 2004). Ehrlich used the term receptors originally called "*Seitenketten*" or side chains on the cell surface which binds antigens. We know now that these side chains are all proteins. A receptor is a protein which binds to a certain chemical molecule or ligand and initiates a cellular response. There are several protein molecules in the plasma membrane which help to transfer the information across the membrane. There are primarily four kinds of membrane receptors.

1. Ligand gated ion channels, bind a specific ligand and opens a channel to allow the transport of chemical components, e.g.: acetylcholine, GABA-A, glutamate receptors
2. Tyrosine kinase receptors, phosphorylate effector molecules upon ligand binding and initiate signaling, e.g.: insulin, growth factor, interferon receptors
3. Guanylate cyclase receptors, couples to guanylate cyclase to initiate the signaling cascade, e.g.: the atrial natriuretic factor receptor
4. G protein-coupled receptors

1.3 G protein-coupled receptors

G protein-coupled receptors (GPCRs) are seven transmembrane receptors coupled to the guanine nucleotide binding proteins. GPCRs constitute the largest class of membrane proteins encoded by about 3% of the human genome (~850). GPCRs have been discovered in phylogenetically diverse organisms ranging from yeast to mammals (Fredriksson *et al*., 2005). G protein-coupled receptors get activated by a variety of stimuli such as photons, ions, lipids, peptides, nucleosides, nucleotides, hormones and neurotransmitters. The signal is transduced across the membranes to guanine nucleotide binding proteins or G proteins. Around 50% of the identified GPCRs respond to smell (olfactory receptors) (Glusman *et al*., 2001). Only a small percentage of the non-olfactory receptors have been pharmacologically targeted till now. Still recent estimates say that about 40-50% of the marketed drugs target these GPCRs (Flower, 1999 *et al*.; Kroeze *et al*., 2003). So the

diversity in the GPCR family and the potential as pharmacological targets necessitates an extensive investigation of these proteins.

The knowledge of GPCRs that we had till 1986, when the first reports of primary structure of β-adrenergic receptors from hamster (Dixon *et al*., 1986) and turkey (Yarden *et al*., 1986) appeared was mainly based on pharmacological studies, radioligand binding studies of these low abundance proteins from natural sources. The identification of the seven transmembrane architecture of bovine rhodopsin similar to bacteriorhodopsin, by electron cryomicroscopy, led to identification and modeling of several GPCRs (Unger *et al*., 1997). More than 1000 GPCRs and putative GPCRs, were submitted to GenBank, to date and the list is increasing, thanks to molecular cloning techniques.

Fig 1.2 shows the key events in the signal transduction cascade through GPCRs. Ligands activate the GPCR, which in turn activates the G proteins on the cytoplamic side of the membrane. Heterotrimeric G proteins after GTP exchange get seperated into α and βγ subunits and activate the downstream effector molecules, which gives rise to a biological response.

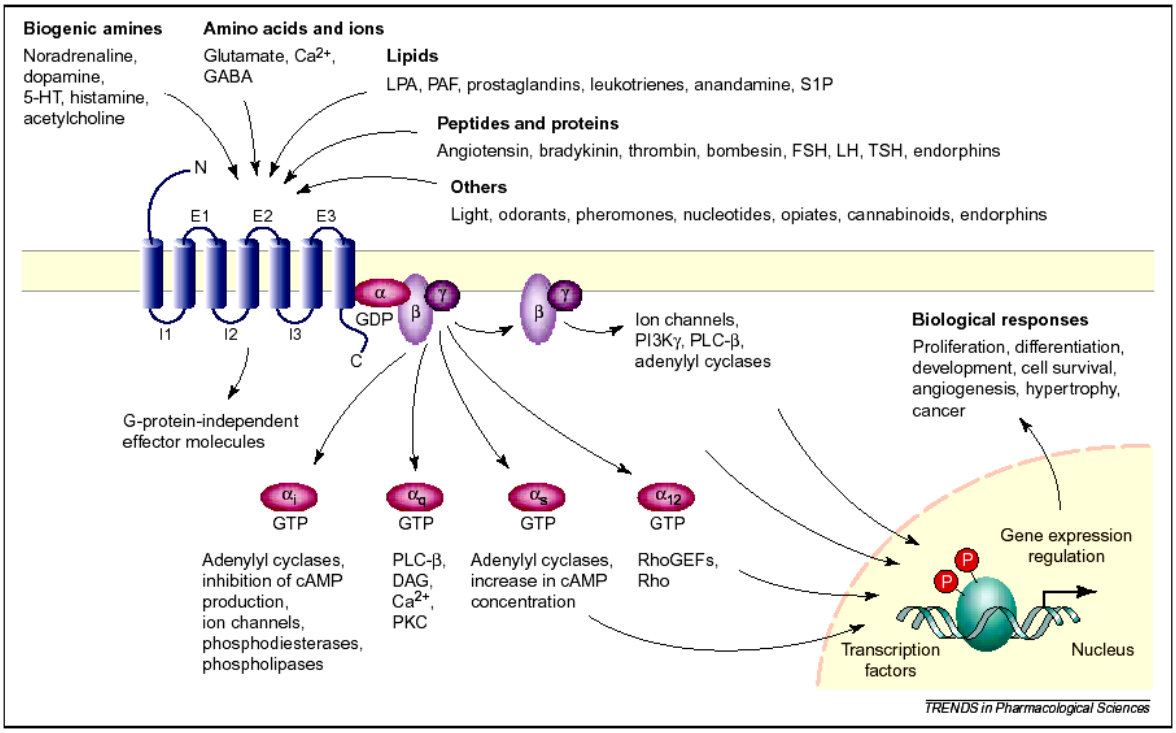


Fig 1.2: Important steps in receptor-G protein mediated signal transduction (Marinissen, 2001).

1.3.1 Classification of GPCRs

Based on the amino acid sequence similarity and nature of ligand, GPCRs are classified into 6 classes in GPCR Data Base (GPCRDB). Around 850 GPCRs are reported to date from the human genome, which are found in the first 3 major classes.

1. Class A or Rhodopsin-like
2. Class B or Secretin-like
3. Class C or metabotropic glutamate / pheromone
4. Class D or Fungal pheromone
5. Class E or cAMP receptors
6. Frizzled / Smoothened family

Rhodopsin-like receptors form the largest receptor class. The overall sequence identity of the receptors in this class is very low. The identity of Class I receptors is determined by a set of 20 amino acids highly conserved in this class and located in the cytoplasmic half of the transmembrane receptor core. These residues are required for protein stability and receptor activation (Wess *et al*., 1993; Baldwin *et al*., 1994; Wess *et al*., 1997). The only residue that is conserved among all Class A receptors (Fig 1.3) is the arginine in the Asp-Arg-Tyr (DRY) motif at the cytoplasmic side of the transmembrane segment, TM3 (Probst *et al*., 1992). Ligands binding to Class A receptors include light, odorants, biogenic amines, protein hormones, peptides, opioids, lipid-like molecules, nucleosides or nucleotides, etc,. In most Class A receptors, a disulphide bridge is connecting the second and third extra cellular loops. In addition, a majority of the receptors have a palmitoylated cysteine in the C-terminal tail forming a putative fourth intracellular loop.

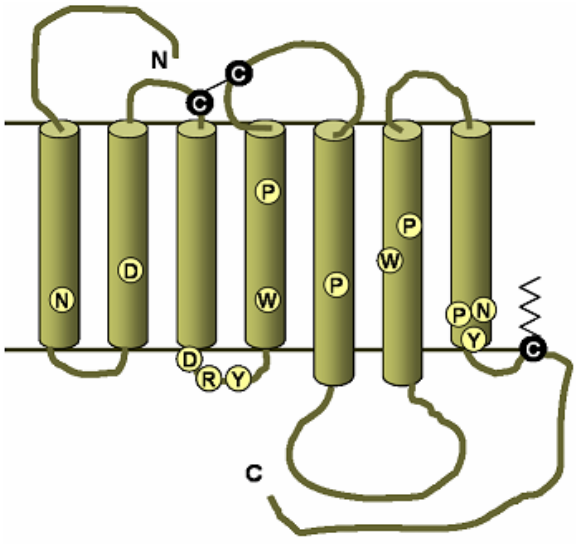


Fig 1.3: Topological model of a prototypical member of Class A GPCRs. The conserved residues are represented by black letters in white circles. The disulphide bridge is represented by crosslinked white C in black circles. N is the N-terminus and C is the C-terminus of the protein. A unique DRY motif is present.

Class B receptors (Fig 1.4) include approximately 20 different receptors for a variety of intestinal peptide hormones and neuropeptides. These receptors contain a relatively large N-terminal extracellular domain with six conserved cysteine residues presumably forming disulphide bridges. Only the disulphide bridge between 2nd and 3rd extracellular loops is the common feature between Class A and Class B receptors. Notable difference is that there is no DRY motif in the Class B receptors (Ulrich *et al*., 1998).

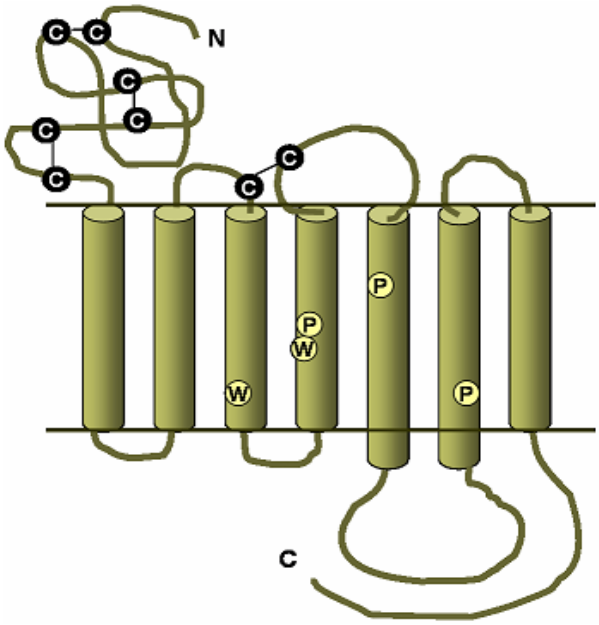


Fig 1.4: Topological model of a prototypical member of Class B GPCRs. Class B receptors have a long N terminus with 6 conserved cysteine residues probably forming disulphide bonds. (Fig 1.3, 1.4 and 1.5 are redrawn from Gether, 2000)

Class C receptors (Fig 1.5) have an exceptionally long amino terminus. The receptors include the metabotropic glutamate receptors, GABA-B receptors (Kaupmann *et al*.,1997), calcium receptors (Brown *et al*., 1993), vomeronasal receptors and mammalian pheromone receptors. Class C receptors like Class A and B receptors, have two putative disulphide forming cysteines in 2nd and 3rd extracellular loops. The N-termini of the metamorphic receptors have certain homology with bacterial periplasmic binding proteins, especially with leucine, isoleucine, and valine binding proteins (O'Hara *et al*., 1993). So it is believed that the amino terminus of Class C receptors contains the ligand binding site (Conn *et al*., 1997; Brown *et al*., 1996).

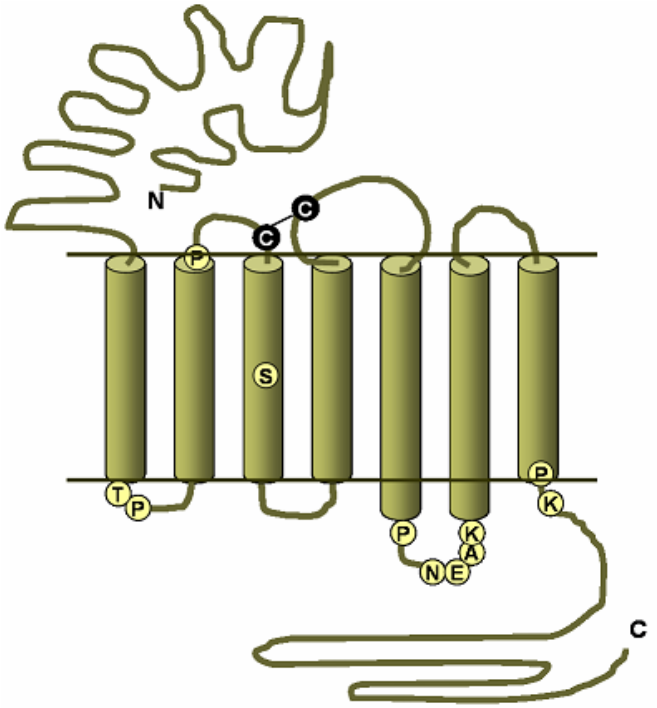


Fig 1.5: Topological model of a prototypical member of Class C GPCRs. Class C receptors have extremely large N termini ranging from 500-600 amino acids. The disulphide bridge is represented by crosslinked white C in black circles. N is the N-terminus and C is the C- terminus of the protein.

Class D receptors are expressed in organisms like yeast, and are functional during mating. Class E receptors are cAMP receptors with higher similarity to the secretin family receptors, discovered in amoeba *Dictyostelium discoideum* and slime mold *Polyspondylium pallidum* (Oyama et al., 1986; Kawabe *et al*., 2002). Frizzled/Smoothed class receptors were discovered in *Drosophila melanogaster* and named after the Frizzled locus contributing to cytoskeletons of the epidermal cells. These receptors are found to regulate cell development, proliferation, differentiation and apoptosis (Wang *et al*., 2006).

1.3.2 Structural features of GPCRs

Not much high resolution structural information is available about GPCRs. Several models were constructed for the GPCRs. Most of these models are based on the x-ray or electron cryomicroscopic structures of bacteriorhodopsin (Henderson *et al*., 2000) and bovine rhodopsin (Unger *et al*., 1997). Bovine rhodopsin is the only GPCR whose structure has been studied by x-ray crystallography till now (Palczewski *et al*., 2000) (Fig 1.6). The initial crystals were of the inactive protein. The active metarhodopsin II form of the receptor was obtained in crystalline form later (Choi *et al*., 2002; Salom *et al*., 2006). In the rhodopsin structure, the arrangement of 7 transmembrane helices relative to each other, is clear now. The 7 transmembrane helices are arranged in a clockwise manner (intracellular view) forming a tightly packed helical bundle (Baldwin *et al*., 1994). The earlier cryo electron microscopic studies showed that helices 1, 2, 3 and 5 are tilted by

about 25 degrees. Helices 4 and 7 are perpendicular to the plane of the membrane. Helix 6 appears almost perpendicular to the plane of the membrane in the cytoplasmic side and tilted towards helix 5 on the extracellular side (Unger *et al*., 1997). The high resolution x-ray structure of bovine rhodopsin showed a so called 8th helix at the cytoplasmic membrane side. The other important finding is that the helices are not straight, but are kinked and bent. Kinks in the helices 1, 4, 5, 6 and 7 were associated with proline residues (Stenkamp *et al*., 2002). Further structural information is needed to understand the mechanism of activation. More and more GPCRs are gaining pharmacological interest and need more structural information to develop potent drugs and to understand the mechanism of action.

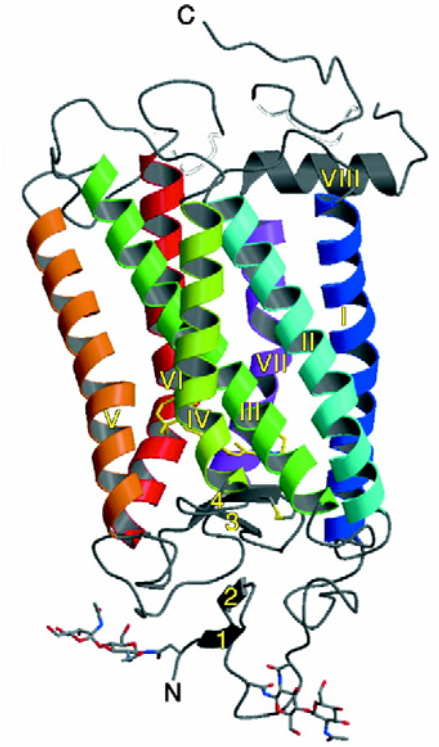


Fig 1.6: 3D Structure of bovine rhodopsin. 7 transmembrane helices are shown as ribbons and numbered in roman letters. N and C are the amino and carboxy termini of the protein (Palczewski *et al.*, 2000).

1.4 Heterotrimeric G proteins

Heterotrimeric G proteins are members of a super family of GTPases which are conserved from bacteria to mammals (Gilman *et al*., 1995). Heterotrimeric G proteins are reported in yeast, plants (Oki *et al*., 2005), invertebrates (insects by Knight *et al*., 2004), and vertebrates. They were initially called N-proteins after their function as nucleotide

binding proteins. Heterotrimeric G proteins transduce the receptor generated signals into the cell (Gilman *et al*., 1987). GPCR signal transduction is mainly carried out by these G proteins though G protein independent pathways are also known. There are three partners in the heterotrimer (Fig 1.7) of G proteins. One subunit each of α, β and γ form a trimer with one Mg^{2+} and one GDP molecule embedded in a pocket of the α subunit.

In humans there are 21 different α-subunits (encoded by 17 genes), some of which are splice variants of the same gene. The molecular weights of these proteins range from 39 to 46 KDa. α-subunits have two distinct domains; a Ras-like GTPase domain and a unique α-helical domain. Based on the amino acid similarity and function, the G proteins can be divided into four main families: $G\alpha_{s,}$ $G\alpha_{i/o,}$ $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Helper *et al*., 1992) (Table 1.1). The $G\alpha_s$ class contains $G\alpha_s$ (several splice variants) and $G\alpha_{olf}$ (specifically expressed in olfactory tissue, Jones *et al*., 1989). This class of proteins activates adenylyl cyclase and increase the production of cAMP. These proteins are substrates for ADP-ribosylation of an argininyl residue catalysed by the A_1 subunit of a cholera toxin, which inhibits the intrinsic GTPase activity of these proteins (Gilman *et al*., 1989).

The $G\alpha_{i/o}$ class contains three subtypes of $G\alpha_i$: i_1, i_2, i_3, two forms of transducin $G\alpha_t$: $G\alpha_{tRod}$ (Rod cell specific) and $G\alpha_{tCone}$ (Cone cell specific), two forms of $G\alpha_o$: $G\alpha_{oA}$ and $G\alpha_{oB}$ (exclusively expressed in brain), $G\alpha_{gust}$ (gustatory epithelium) and $G\alpha_z$. Except for $G\alpha_{z,}$ all members of this family have a conserved C-terminal cysteine, which is the site of ADP-ribosylation by pertussis toxin. This modification uncouples the G proteins from the corresponding receptors (Ui *et al*., 1990). $G\alpha_i$ family members inhibit the adenylyl cyclase and decrease the cAMP production (Taussig *et al*., 1994). $G\alpha_t$ activates the cGMP phosphodiesterase in the retina. These proteins activate the potassium channels and decrease the calcium channels and chloride channels. Phospholipase A_2 is another effector for this family members. $G\alpha_{q/11}$ family contains $G\alpha_q$, $G\alpha_{11}$,$G\alpha_{14}$ and $G\alpha_{16}$. These proteins activate phospholipase C (Helper *et al*., 1993), protein kinase C and calcium channels. $G\alpha_{12}$ and $G\alpha_{13}$ constitute the last family. These proteins interact with mitogen activated protein kinases (MAPK) through Rho proteins. They are important in the cytoskeleton formation and other functions during the cell differentiation (Jho *et al*., 1997).

Table 1.1 Effectors for different G protein subunits. Red arrows indicate an increased activity and green arrows indicate a decreased activity (Morris *et al*., 1999; Wettschureck *et al*., 2005).

$G\alpha_s$	Adenylyl cyclase	↑
	Na^+ channels	↑
	Cl^- channels	↑
$G\alpha_{i/o}$	K^+ channels	↑
	Ca^{2+} channels	↓
	Na^+ channels	↓
	Cl^- channels	↓
	Adenylyl cyclase	↓
	cGMP phosphodiesterase ($G\alpha_t$)	↑
$G\alpha_{q/11}$	Phospholipase C	↑
$G\alpha_{12/13}$	Rho GEFs	↑
$G\beta\gamma$	Adenylyl cyclase I	↑
	Adenylyl cyclase II, IV	↓
	Phospholipase C	↑
	Phosphoinositol 3-kinase	↑
	Phospholipase A2	↑
	K^+ channels	↑
	Ca^{2+} channels	↓

Six different G protein β and 12 different γ subunits have been reported in humans till now (Clapham *et al*., 1997). The G_β subunits have a molecular mass of approximately 35 KDa. The amino acid sequences of these proteins contain 7 or 8 tandem repeats with a central conserved Trp-Asp (WD) sequence that is termed a WD-40 motif (Garcia-Higuera *et al*., 1998). The γ subunits are small ranging from 7-8.5 KDa. The C-termini of all γ subunits contain the sequence CAAX, where A can be any aliphatic amino acid. The protein undergoes a post-translational prenylation at the Cys in this sequence followed by proteolytic cleavage of the last three amino acids (Backlund *et al*., 1990). γ1 subunit is unique in two ways, that it is specific for the transducin heterotrimer and that the prenyl group attached to the C-terminus is a farnesyl group. All other γ subunits are modified by geranyl geranyl groups (Mumby *et al*., 1990). β and γ form a stable tight dimer complex. The βγ dimer also interacts with a wide range of effector molecules (Clapham *et al*., 1997). A domain on β subunit interacting with several effector molecules was identified earlier

(Yan *et al*., 1996). γ subunits were reported to be playing an important role in the G protein interaction with the receptors (Azpiazu *et al*., 2001; Kisselev *et al*., 2006). There is also increasing number of reports available which confirm that γ subunit is involved in the βγ interaction with effector molecules like phospholipase C (Akgoz *et al*., 2002). There is evidence that the prenyl modification of the γ subunit is a requirement for the βγ complex action on effectors (Katz *et al*., 1992). Proper processing of G protein γ has been reported to be dependent on complex formation with a β subunit (Pronin *et al*., 1993).

1.4.1 Structural features of G proteins

X-ray crystallographic structures of $G\alpha_t$ (Noel *et al*., 1993), $G\alpha_{i1}$ (Coleman *et al*., 1994), chimeric proteins *i.e.* $G\alpha_{i/12}$ and $G\alpha_{i/13}$ (Kreutz *et al*., 2006) and $G\alpha_{i1/q}$ (Tesmer *et al*., 2005) have been reported till now with different nucleotides bound to them and with effector molecules. $G\alpha_{i1}$ was crystallized together with $\beta_1\gamma_2$ (Wall *et al*., 1995, Fig 1.7). Chimeric $G\alpha_{t/i}$ and $G\alpha_t$ together with $\beta_1\gamma_2$ were also crystallized (Sondek *et al*. , 1996). There are distinct conformational changes in the α subunit but not in βγ subunit. There are three conformational changes with the so-called switch regions in the α subunit. The N-terminus is of α-helical secondary structure and protrudes away from the two domains. The α-helical domain consists of one bigger α-helix in the centre surrounded by five shorter helices. In the Ras like GTPase domain a 6-stranded β sheet is surrounded by six helices of this domain and one helix from the helical domain. In the βγ dimer the γ subunit entwines with the N-terminal helix of β subunit and touches 6 of 7 propeller blades. This dimer forms a tight complex.

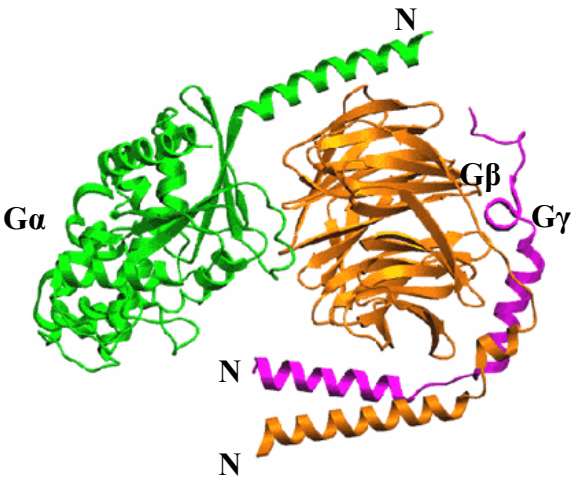


Fig 1.7: Structure of the G protein heterotrimer. Subunit in green is Gα, golden yellow is Gβ and purple is Gγ. N stands for the N-terminus of the protein. http://www.fli-leibniz.de/IMAGE.html

1.5 Mechanism of signal transduction

Our present knowledge of what happens when the ligand binds to the receptor is very limited and based on the rhodopsin crystal structures, in active and inactive forms. Models have been constructed based on the TM conformational changes observed in rhodopsin (Fig 1.8). When a ligand binds to the receptor the transmembrane helices undergo a relative orientation change to each other. In rhodopsin TM3 and TM7 are constrained by a salt bridge in the inactive form (Govardhan *et al*., 1994). When the ligand activates the receptor the salt bridge is broken and TM3 rotates and moves apart relatively more on the cytoplasmic side (Fahmy *et al*., 1995). Considerable evidence indicates that TM3 and TM6 move together leaving a cavity on the cytoplasmic side which might be the activation site for the G proteins (Farrens *et al*., 1996).

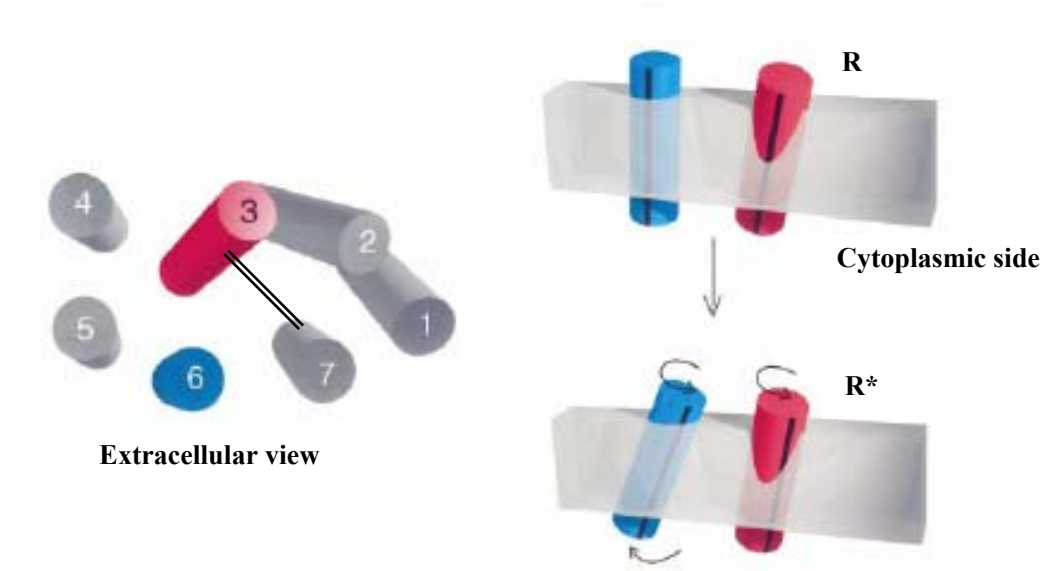


Fig 1.8: Arrangement of 7 transmembranes and conformational changes. The arrangement is based on the projection map of 2D crystals of rhodopsin. R is the inactive form of the receptor and R* is the active form. The double line between TM3 and TM7 represents the predicted salt bridge (based on Unger, 1997).

The invariably conserved arginine in the DRY motif is constrained in a hydrophilic pocket in the inactive form. In the active form this arginine shifts out of the polar pocket because of the protonation of preceeding aspartic acid (Arnis *et al*., 1994) residue leading to cytoplasmic exposure of the buried residues in the crevice formed because of the movement of TM3 helix (Scheer *et al*., 1997). The arginine is a crucial residue here, mutation of which allows coupling to G proteins but no activation (Acharya and Karnik., 1996).

The C-terminal helix of the G protein is supposed to be interacting with the amino acids in the crevice formed in the receptor. The message is passed onto the helical domain and then to the GTPase domain, which activates the G proteins attached to the receptor. As shown in fig 1.9, activation of G proteins, leads to changes in the three switch regions in the GTPase domain. GDP is held at the interface of the two domains of the α subunit. Activated switches loosen the GDP binding. All these switches are in the interface between the α and βγ subunits. GDP is then exchanged for GTP. Guanine nucleotide exchange factors assist in this exchange. The three switches are held in place by contacts to the terminal γ phosphate (Coleman, 1994). The altered switch regions decrease the interactions with the βγ subunits thereby dissociating the whole trimer complex into two functional units, the α subunit and the βγ dimer.

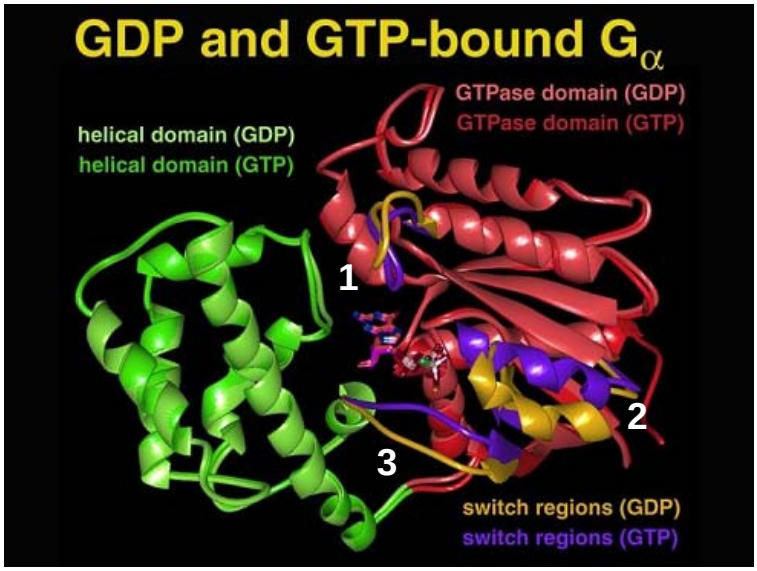


Fig 1.9: Active and inactive conformations of Gα subunits overlapped on each other. The helical domain is shown in green colour (light colour for GDP bound form and dark for GTP bound form) and the GTPase domain in pink colour. The three numbered switch regions are represented in yellow when bound to GDP and in violet when bound to GTP. http://www.bmb.psu.edu/faculty/tan/lab/gallery/galpha_ribbon3.jpg

Both functional units activate the respective effector molecules to pass on the signal downstream. The Gα subunit has an intrinsic GTPase activity and so the bound GTP is hydrolysed to GDP, which converts the G protein again to the inactive form. The hydrolysis is assisted by GTPase activation proteins (GAPs). The trimer complex forms again.

1.6 Cannabinoid receptors

Two types of cannabinoid receptors have been identified so far. Cannabinoid receptors were identified in the rat brain in the year 1988 (Devane *et al*., 1988). Cannabinoid receptor 1 (CB1) was cloned in the year 1990 (Matsuda *et al*., 1990) and cannabinoid receptor 2 in 1993 (Munro *et al*., 1993). A splice variant of CB1, CB1a has also been isolated (Rinaldi-Carmona *et al*., 1996). CB1 receptors show a high level of sequence identity between the different mammalian species: human to mouse 90% and human to rat 96% (Chakrabarti *et al*., 1995).

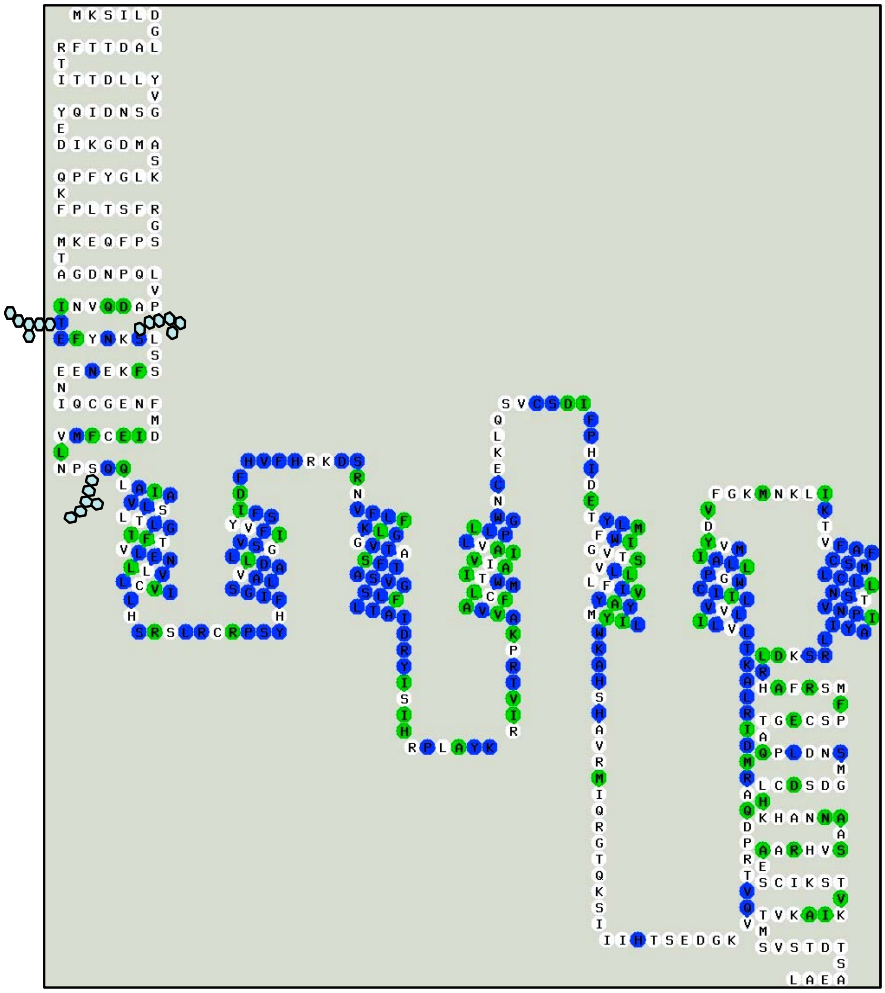


Fig 1.10: Secondary structure model of CB1 receptor showing the conserved residues with CB2. Blue circles indicate the conserved residues between CB1 and CB2 residues. Green circles indicate the amino acids unique to the CB1 receptor. Linked hexagons represent the N-linked glycosylation sites. CB1 and CB2 share only 44% overall identity but around 68% identity in the transmembrane region. (image based on snake like plots from GPCRDB)

CB2 receptors show more interspecies differences. CB1 receptors are expressed in central nervous system and also in peripheral tissues including pituitary gland, immune cells, and reproductive tissues and in sperm cells, lung, adrenal gland, etc, (Pertwee *et al*., 1997). CB2 receptors are expressed mainly in immune cells like B cells and NK cells (Galiegue *et al*.,1995). Human CB1 and CB2 receptors share a overall identity of 44% but the transmembrane regions have around 68% identity (Fig 1.10). Transmembrane segments 4 and 5 show a high degree of dissimilarity which might be the important site for the ligand selectivity.

1.6.1 Structural features of cannabinoid receptors

Cannabinoid receptor 1 consists of 472 amino acids and cannabinoid receptor 2 is a polypeptide of 360 amino acids. These receptors have 7 transmembrane helices and a juxta membrane 8th helix (Qun Xie *et al*., 2005). These proteins don't have a specific N-terminal signal peptide. There are three potential N-glycosylation sites (NIT, NKS, NPS) on the N-terminus of CB1 receptor, but they are not important for ligand binding (Howlett, 1991) and one site on CB2 receptor (NGS).

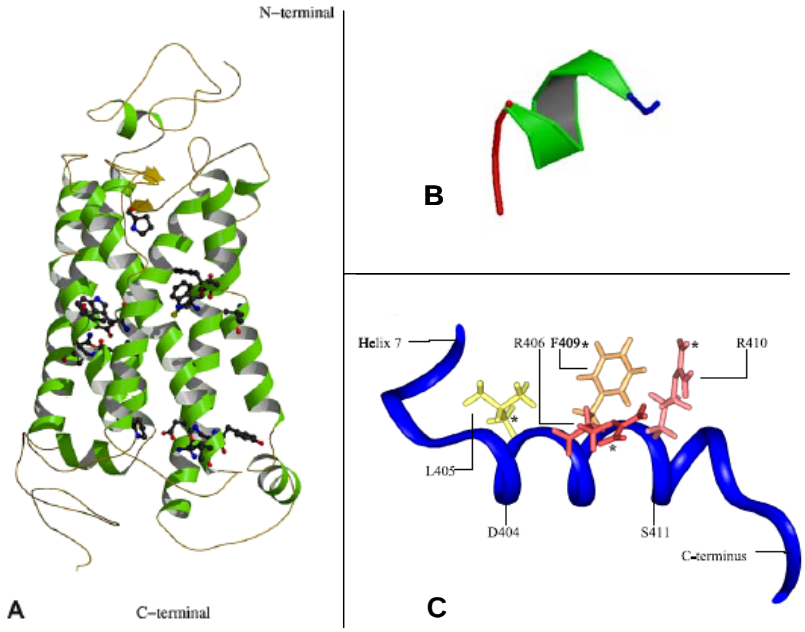


Fig 1.11: A. Homology model CB1 receptor. α helices are shown as Green ribbons. The conserved residues are represented as ball and stick models (Montero, 2004). **B. ic3 loop of CB1 receptor, D^{338}-V^{346}.** NMR structure of third intracellular loop bound to Gi1 protein (Ulfers *et al.,* 2002). **C. 8th helix of CB1 receptor, I^{397}-G^{418}.** The 8th helix or the 4th intracellular loop of CB1 receptor was determined by NMR (Choi *et al*., 2004).

The structure of these receptors is not understood in detail. Synthetic CB2 receptor fragments are studied by NMR technique. Recent work by Zheng *et al*, 2006 reported the structure of the first and second transmembrane helices of the CB2 receptor (27-101 amino acids). Zhao *et al.* (2006) reported the structure of TM5 and the third intracellular loop (180-233 amino acids) also by NMR technique. Qun Xie *et al.* (2005) solved the structure of 8th membrane parallel helix of the CB1 (I^{397}-G^{418}) and CB2 (I^{298}-K^{319}) receptors. They also reported that Cys^{416} in CB1 and Cys^{313} in CB2 point towards the membrane suggesting a possible palmitoylation at these conserved residues. The structure of the third intracellular loop (ic3) of CB1 (D^{338}-V^{346}), was determined bound to $G\alpha_{i1}$. This report confirms that the ic3 forms an α helix (Ulfers *et al*., 2002).

1.6.2 Cannabinoid ligands and ligand binding site

Cannabinoid receptors are now known to be the site of action for the active compounds of marihuana like Δ^9–tetra-hydrocannabinol (Δ^9-THC). Cannabidiol (CBD) and cannabinol are other abundant natural cannabinoids active at these receptors (Fig 1.12). These are the active compounds in the prevalent, ancient street drug Marijuana (*Cannabis sativa*). Δ^9 –THC has an almost equal affinity for both CB1 and CB2 receptors (Huffmann *et al*., 2000). In 1992 a ligand for cannabinoid receptors was isolated from pig brain. This endogenous cannabinoid ligand was arachidonoyl ethanolamide, a derivative of arachidonic acid and named as anandamide after the Sanskrit word *ananda* meaning *bliss* (Devane *et al*., 1992). Another endocannabinoid is 2-arachidonoyl glycerol or 2-AG, which is more abundant but less potent than Δ^9 –THC (Sugiura *et al*., 2000). Most of the endogenous cannabinoids discovered so far are high or low efficacy agonists. But there is one recently reported inverse agonist called virodhamine after the Sanskrit word *virodh* meaning *oppose* (Pertwee *et al*., 2005). There is an extensive list of pharmacological benefits of cannabis dating from 2000 BC.

High affinity non-eicosanoid, non classical cannabinoids were first developed by the pharmaceutical company, Pfizer. Most important and potent among them is CP 55940. Another important and extensively used ligand is WIN 55,212-2, developed by a Sterling Winthrop research team. The break through selective ligands for CB1 and CB2 receptors were developed by Sanofi. Both these ligands are antagonists or inverse agonists.

SR141716A is a selective antagonist for the CB1 receptor and SR144528 is selective for the CB2 receptor (Shire *et al*., 1999). Most of the cannabinoid ligands are highly lipophilic and nearly insoluble in water. One exception and worth mentioning is the ligand O-1057 which is highly water soluble and almost as potent as CP55940 (Pertwee *et al*., 2000).

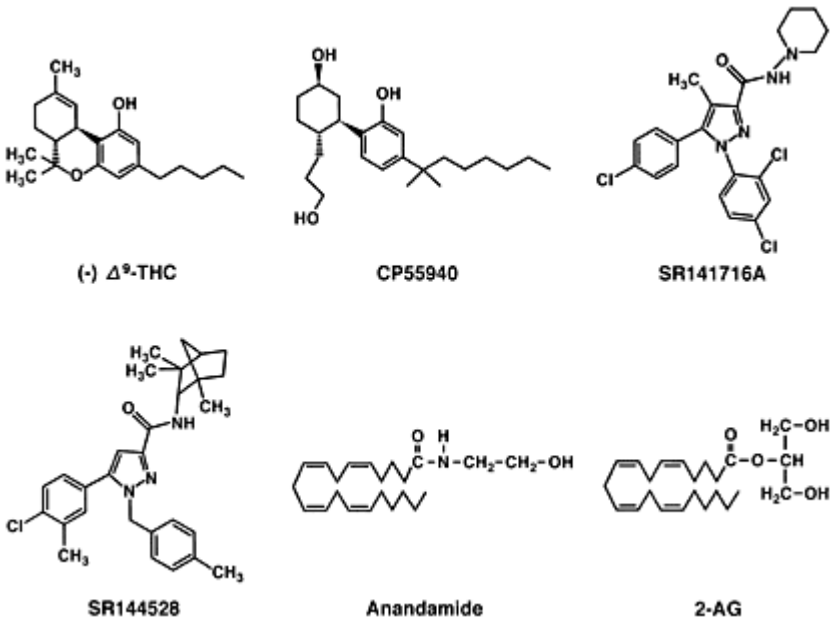


Fig 1.12: Some commonly used ligands of cannabinoid receptors. Δ^9 –THC is the active constituent of *Cannabis*. CP55940 is the first non-classical ligand synthesized. Anandamide and 2-AG are endocannabinoids. SR141716A and SR144528 are CB1 and CB2 selective antagonists developed by Sanofi-Aventis.

Different amino acid residues from transmembranes 3, 4, 5 and 6 were identified to be important for the binding of different cannabinoid ligands. Lysine K^{192} of CB1 receptor (K^{109} of CB2 residue) was found to alter the binding of several agonists but not for WIN55,212-2 (Chin *et al*., 1998). This result proved that the binding site is different for this ligand. An aromatic microdomain modeled from the residues F^{190} (TM3), F^{201} (TM3), W^{256} (TM4), W^{280} (TM5) and W^{357} (TM6) was shown to form a binding site for many ligands. Mutation of these residues showed a profound effect on ligand binding. Mutation of F190A reduced the affinity for the agonist anandamide, whereas the mutations F^{201}A, W^{280}A and W^{357}A reduced the affinity for ligands like WIN55,212-2 and SR141716A (McAllister *et al*., 2003).

1.6.3 Signal transduction by cannabinoid receptors

1.6.3.1 Receptor-G protein interactions

Cannabinoid receptors interact with $G\alpha_{i/o}$ proteins. A reconstituted system having Sf9 cell membranes expressing CB1 and CB2 receptors and $G_{i/o}$ trimers from bovine cortex shows that CB1 and CB2 receptors interact equally with G_i protein and less effective at G_o protein. CB2 was less efficient than CB1 to bind to G_o proteins (Glass *et al*., 1999), in a ligand dependent manner. Mukhopadhyay *et al*. (2001) demonstrated that distinct intracellular domains determine G protein subtype selectivity of CB1 receptor. The so called 8th helix (CB1$^{401-417}$) peptide reduced the CB1 receptor association with $G\alpha_{i3}$ but not $G\alpha_{i1}$ and $G\alpha_{i2.}$ Peptide from the third intracellular loop reduced the association of CB1 receptor with $G\alpha_{i1}$ and $G\alpha_{i2}$ but not $G\alpha_{i3.}$ So these intracellular receptor regions are important for G protein interaction. CB1 receptor is a constitutively active receptor (R*G) and is also able to sequester $G_{i/o}$ proteins in a inactive conformation (IARºG) making other receptors, which share these proteins, inactive (Vasquez *et al*., 1999). The receptor-G protein complexes involved in these phenomenon are explained by a cubic ternary model

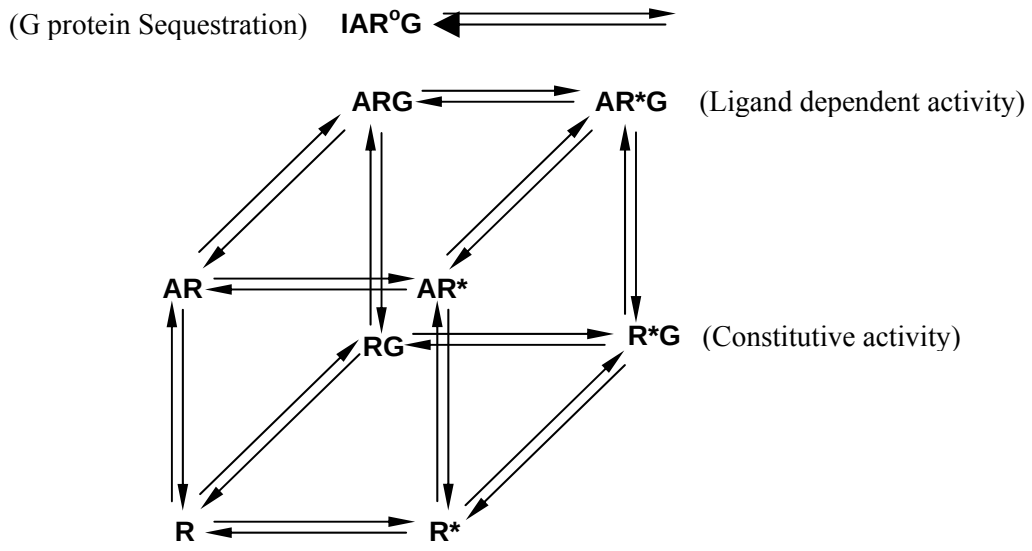


Fig 1.13: Cubic ternary complex model of Ligand-Receptor-G protein. R represents the inactive form of receptor and R* the active form. G is the G protein. A is the agonist and IA is the antagonist. The R*G form is responsible for the constitutive activity of the receptor which is shifted towards the AR*G in the presence of agonist. In the presence of inverse agonist the inactive ternary complex ARG is stabilized and G proteins are sequestered from the pool. (Howlett *et al*., 2004).

as in Fig 1.13. Truncation of the distal C-terminal tail of the receptor (CB1-417) enhanced both the constitutive and sequestration ability. Nie *et al.* (2001) reported that mutation of aspartate (D164N) in the second transmembrane segment of CB1 abolished both these abilities of the receptor without disrupting agonist stimulated activity. Receptors interact with G proteins in a ligand dependent manner. Inverse agonists like SR141716A seem to stabilize the inactive IAR^oG state of the complex which leads to sequestration (Howlett *et al*., 2004).

1.6.3.2 Adenylyl cyclase mediated signaling

Agonist activated receptor induces the activation of $G_{i/o}$ proteins. The G_i subunits inhibit a few isotypes of adenylyl cyclases AC V and AC VI (Dessauer *et al*., 2002). G_o subunits inhibit isotypes AC I, and G_z subunits inhibit AC I and AC V (Taussig *et al*., 1995). Inhibition by G_i proteins is characteristic of cannabinoid agonists in the brain tissue (Childers *et al*., 1994). The decreased cAMP concentration reduces the cAMP dependent protein kinase (PKA) activity which in turn causes a decreased dephosphorylation level of potassium channels and increased potassium currents (Childers *et al*., 1996) leading to hyperpolarization of the membranes. The CB2 receptor also shows this kind of inhibitory effect on cAMP production in immune cells (Felder *et al*., 1995). Cannabinoid receptors not only inhibit cAMP production but also show the contrary effect in certain cases. This effect depends on the cell type and the available AC subtype population. In cells having high levels of AC II, AC IV and AC VII, cannabinoid receptor activation leads to an increase in the cAMP levels but not because of the G_i interaction but because of $\beta\gamma$ interaction with these subtypes (Rhee *et al*., 1998). A recently studied mechanism is the direct interaction of cannabinoid receptors in the presence of dopamine receptors with the G_s protein to increase the cAMP production (Jarrahian *et al*., 2004).

1.6.3.3 Regulation of ion channels

Cannabinoid agonists modulate several ion channel activities. N-type voltage gated Ca^{2+} channels are inhibited through the $G_{i/o}$ proteins (Fig 1.14, Guo *et al*., 2004). Q type calcium currents were also inhibited as a result of cannabinoid receptor activation (Mackie *et al*., 1995). Inwardly rectifying potassium channels were activated by cannabinoid

receptor activation (Fig 1.14, Guo *et al*., 2004). This mediation was G protein dependent but not dependent on cAMP levels. This indicates that βγ subunits are involved which alter the IK.ACh type of potassium channels. These physiological mechanisms in the neurons attribute to the neuroprotective function of cannabinoid receptors by a mechanism called depolarization-induced suppression of inhibiton (DSI) or excitation (DSE). According to this mechanism the depolarization opens the N-type Ca^{2+} channels, which leads to endocannabinoid production. Diffusion of endocannabinoids from the postsynaptic neuron stimulates the CB1 receptors on presynaptic terminals leading to decreased release of neurotransmitters like GABA (Wilson *et al*., 2002). Several other mechanisms of signaling by cannabinoid receptors like MAPKinase pathway, Jun-N-terminal kinases, Nitric Oxide, etc were reviewed in Howlett (2005).

CB2 receptors are known to modulate the ion channel activity, but less is known about the physiology of CB2 receptors. There are reports that CB1 receptors sometimes don't utilize G proteins as transducers. Sanchez *et al.* (2001) showed that spingomyelinase activation by the CB1 receptor was mediated by the adaptor protein Fan but not by G proteins.

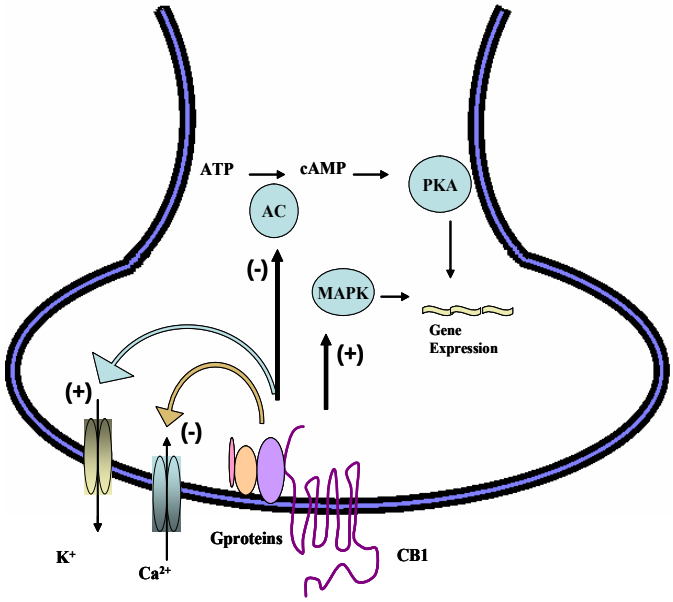


Fig 1.14: Signaling pathway of CB1 receptor by agonists. Activated receptor stimulates the $G_{i/o}$ proteins which inhibit the adenylate cyclase activity and subsequent inactivation of protein kinase A (PKA) or to stimulation of mitogen activated protein kinase (MAPK). CB1 activation is inversely coupled to voltage activated Ca^{2+} channels and stimulation of inwardly rectifying K^+ channels, which subsequently inhibit neurotransmitter release at the neuronal ends. (redrawn from Di Marzo, 2004)

1.6.4 Cannabinoid receptor interactions with other proteins

1.6.4.1 Receptor dimerization

GPCR dimerization is an accepted phenomenon now-a-days with more and more reports confirming this once not accepted fact. GPCRs form homo and heterodimers. Heterodimerization leads to binding sites that bind ligands not recognized by either components. The heterodimerization also could change the signaling process of the component proteins (Mackie *et al*., 2005). CB1 receptors do form homo and heterodimers. The existence of homodimers has been confirmed by a "dimer antibody" which only recognizes a dimer but not a monomer and also by classical immuno-precipitation (Wager-Miller *et al*., 2002). CB1 and D2 dopamine receptors together form heterodimers (Kearn *et al*., 2004). The functional significance of this heterodimer formation was demonstrated by Glass and Felder (1997). In general both these proteins decrease the cAMP production. But when both proteins are expressed together, high concentrations of CB1 agonists increase cAMP production making D2 agonists inefficient. CB1 and opioid receptors also were reported to form heterodimers with each other. A recent report demonstrates that all the three opioid receptor subtypes (μ, κ and δ) directly interact with the CB1 receptors. CB1 receptor agonists attenuated the opioid receptor mediated signaling reciprocally.

1.6.4.2 Cannabinoid receptor desensitization and internalization

GRK3 and β-arrestin 2 mediate agonist dependent CB1 receptor desensitization. The C-terminal residues 418- 439 were found to be important for this desensitization (Jin *et al*., 1999). The residues S^{426} and S^{430} seem to be the most likely sites of phosphorylation by GRK3. It is reported that β-arrestin does not interact with these two Ser residues, since the mutant receptors also internalized normally. The exact site of interaction with arrestin is not clear yet.

1.6.5 Functions of cannabinoid receptors

Cannabinoid receptor distribution symbolizes their functions in the body. CB1 receptors are mainly distributed in the central and peripheral nervous system. The coupling

of these receptors to the ion channels makes the endocannabinoid system ideal for modulating neurotransmitter release. Inhibition of glutamatergic, GABAergic, glycinergic, cholinergic, noradrenergic and serotonergic neurotransmission has been observed in the central nervous system. In the peripheral nervous system, the CB1 receptor mediates the inhibition of adrenergic, cholinergic, and sensory neuroeffector transmission (Szabo *et al*., 2005). The suppression mechanism observed in the nociceptive neurons correlates with the nociceptive effects of cannabinoids (Walker *et al*., 2005). CB1 receptors are generally credited as neuroprotective, because of this neurotransmitter inhibitory mechanism. Activation of CB1 receptors expressed in basal ganglia and cerebellum modulate the locomotor activity. The cannabinoid receptors in the hippocampus control the short-term memory. The cannabinoid receptor in the neocortex is involved in drowsiness after intoxication. One of the most important effects of cannabis consumption is the increase of appetite, which is controlled by receptors in the hypothalamus (reviewed by Iversen *et al*., 2003). CB2 is predominantly expressed in the immune cells and controls humoral and cell mediated immunity. Cytokine release is modulated by the CB2 receptors. A recent finding is that CB2 receptor maintains the bone mass and protects from osteoporosis (Ofek *et al*., 2006).

1.6.6 Cannabinoid receptors as therapeutic targets

Cannabinoid receptors were targeted for pain relief mainly because of their antinociceptive action. Sativex®, a cannabis based drug containing Δ^9-THC and CBD was approved in Canada for the treatment of neuropathic pain in multiple sclerosis. Marinol® and Cesamet®, containing Δ^9-THC and the analogue nabilone are marketed as medicines for suppressing nausea and vomiting caused in chemotherapy and also to stimulate appetite in AIDS patients. CB1 receptor agonists have many other potential uses in Parkinson's disease, Alzheimer's disease, inflammation (CB2 agonists), fertility, epilepsy and alcohol withdrawal (Robson *et al*., 2005; Pertwee *et al*., 2006). Since the discovery of receptor selective antagonists like SR141716A for CB1 receptor by Sanofi, their use as medicines is also under extensive study. SR141716A (Rimonabant) is approved under the tradename Acomplia® in the European Union, as an anti-obesity drug because of its reversal of the munchis effect of cannabis consumption. Munchis effect arouses the desire to eat sweet foodstuffs.

1.6.7 Purification of cannabinoid receptors

Determination of protein structure in detail is possible by 2D or 3D crystallography and NMR spectroscopy for smaller proteins. All these techniques need milligram quantities of protein. The structure of the cannabinoid receptor has not been determined, as is the case with any other GPCR, except bovine rhodopsin. Cannabinoid receptors or GPCRs are produced in a very low quantity in natural tissues. Purification of these proteins in the quantities required for structural identification is highly tedious or nearly impossible. Several groups have tried to produce these receptors using heterologous expression systems. A few early reports are : Cannabinoid receptor 1, was expressed in insect cells (3.7 pmol/mg, Pettit *et al*., 1994) and COS-3 cells (18.7 pmol/mg, Shire *et al*., 1996). Glass *et al.* (1999) reported expression levels of CB1 and CB2 receptors as 15 and 33 pmol/mg respectively, in insect cells.

The Grisshammer group was one of the first to attempt to produce the cannabinoid receptors in a prokaryotic expression system. The *E. coli* expression system was chosen because of its simplicity, ease of handling and the high productivity obtained. Production of both CB1 and CB2 receptors, was tried as a fusion protein with the maltose binding protein (MBP). The CB2 receptor could be expressed in higher yields (38 pmol/mg) in a functional form and the CB1 receptor could not be expressed even in the detectable limits. The CB1 receptor was highly degraded (Calandra *et al*., 1997). Recently the CB2 receptor was produced and purified in a functional form from *E. coli* (Yeliseev *et al*., 2005). A mixture of 0.5% CHAPS+1% DM+ 0.1% CHS was used to solubilise the protein. The authors reported that they could purify CB2 receptor upto 80-90% purity in small scales (as judged by SDS-PAGE), but large scale purifications yielded only 50-60% pure protein. Additional purification steps improved purity to a small extent (85-90%), but loss of protein was observed because of increased number of purification steps. This purity is not sufficient for crystallization attempts. The CB2 receptor fragments (residues 27-101, residues 180-233) were expressed in *E. coli* for NMR spectroscopic structural determination.

CB1 and CB2 receptors were expressed in functional form in the *P. pastoris* membranes. Purification attempts of polyhistidine tagged receptors were made for both proteins solubilised by 1% DM. The CB2 receptor was bound very tightly to the matrix, which needed upto 500 mM imidazole to elute the protein from the IMAC column, on the other hand CB1 was so loosely bound that the receptor was washed away even with 30 mM imidazole. It has been concluded that the purification of CB1 (Kim *et al*., 2004) and CB2 (Feng *et al*., 2002) was successful in scales suitable for mass spectroscopic analysis. This is quite far from the amount needed for crystallization. They also report that they couldn't detect any ligand binding of the purified proteins. In the literature there are several reports regarding the production of cannabinoid receptors in insect cells using the baculovirus expression system. Purification attempts were made by Makriyannis group, to use the protein again in mass spectroscopic analysis. The CB2 receptor was expressed at a level of upto 9.3 pmol/mg of membrane protein (Filppula *et al*., 2004). CB1 expression levels were much higher with upto 24.5 pmol/mg for SR141716A but only 1.7 pmol/mg for agonist CP55940, which could mean that most of the protein is present in an inactive form not recognized by agonists (Xu *et al*., 2005). Harsh washing steps (with 8M Urea) were used for the purification of the receptors. In these studies they observed several bands in SDS-PAGE after purification which correspond to cannabinoid receptors. Some of them were oligomeric forms and some were N-terminally degraded forms.

1.7 Expression systems for the production of recombinant protein

The ability to clone a gene into a plasmid made it possible to produce a protein of interest in a different organism. If the gene can be expressed in higher yields compared to the natural tissue or cell, it is called overexpression. There are several expression systems being used now-a-days to overproduce proteins. A gene is cloned downstream of an inducible promoter. The expression host can be a prokaryote or an eukaryote. Prokaryotic expression systems are the cheapest and fastest of all expression systems. Another advantage of these systems is the success rate and high yield. Eukaryotic expression systems are relatively expensive and time consuming. Cell-free expression is another novel method to produce recombinant proteins.

1.7.1 Prokaryotic expression system

E. coli is the most frequently used organism for laboratory use, since the first report that it is possible to express foreign genes in *E. coli*. Several promoters are in use like the lac promoter (lactose promoter), the tac promoter (a hybrid of trp and lac promoter), the ara promoter (arabinose promoter) or the bacteriophage T5 or T7 promoters. The T7 promoter system is most successful and most used now-a-days. In this system the gene of interest is under the control of T7 promoter which is recognized by T7 RNA polymerase. The production of T7 RNA polymerase is under the control of lac promoter with a lac operator in between. Under normal conditions the lac repressor, product of the lacI gene suppresses the production of T7 RNA polymerase thereby inhibiting transcription of the gene of interest. Upon addition of IPTG which is a derepressor, T7 RNA polymerase is produced and transcribes the gene of interest using T7 promoter leading to protein production. For the production of recombinant GPCRs in *E. coli*, the tac promoter was used extensively. GPCRs like the β_2 and β_1 adrenergic receptors, the serotonin receptor $5HT_{1A}$, the endothelin receptor-B, the neutotensin receptor, the adenosine receptor A1 and A2a, the neurokinin receptor-2, opioid receptors (Sarramegna *et al*., 2003) etc. and recently the cannabinoid receptor-2 were expressed in E. coli.

1.7.2 Eukaryotic expression systems

There are several drawbacks in prokaryotic expression systems despite their extensive use for protein production. *E. coli* is a simple organism with a simple machinery for protein production and modifications. The post-translational modifications observed in eukaryotic proteins are not seen in prokaryotic proteins and so *E. coli* lack this machinery. Such eukaryotic proteins, produced in *E. coli* might be non-functional or don't express at all. These problems occur mostly with mammalian proteins which have complicated post-translational modifications like glycosylation, acylation and phosphorylation. To overcome these problems several eukaryotic expression systems were developed.

1.7.2.1 Yeasts as expression hosts

Yeasts are the simplest among eukaryotes used for protein production. Yeasts have short generation times (2h), grow on simple media and like bacteria are easy to manipulate. Unlike bacteria they have a complex machinery for most of the post-translational modifications. Several yeast species have been developed as expression hosts. *Saccharomyces cerevisiae* is the first yeast used for heterologous protein production. The inducible GAL1 (galactose inducible) promoter is commonly used. Another most extensively used yeast is the methylotrophic yeast *Pichia pastoris*. *P. pastoris* possesses a strong inducible alcohol oxidase promoter (AOX1) which can be used to induce protein production. The gene of interest is stably integrated into the genome. Multicopy gene integration enhances protein production. Membrane protein production could be increased by using the *S. cerevisiae* α-factor mating signal peptide. It is estimated that alcohol oxidase can constitute upto 80% of the total cell protein (Tschopp *et al.*, 1987; Cregg *et al*., 2000; Reilander *et al*., 1998). Though this system has been successful in producing several proteins in milligram quantities, it too has some drawbacks, when it is used for production of mammalian proteins like GPCRs. The N-glycans added by yeasts are different from those of the mammalian cells, which might affect the glycosylation status and there by the activity of the protein produced (Eckart *et al*., 1996). The lipid composition of the yeast membranes is also different from that of the mammalian cells. Yeasts do not produce cholesterol which is one of the important lipids of the mammalian cell membrane. Other commonly used yeast species are *Schizosaccharomyces pombe*, *Hansenula polymorpha* and *Candida boidinii* (Gellissen *et al*., 2000). GPCRs like the β_2 adrenergic receptor (115 pmol/mg), the μ-opioid receptor (100 pmol/mg), the bovine rhodopsin (2 mg/10^{10} cells), the endothelin receptor-B (30-60 pmol/mg), were produced in high levels (Sarramegna *et al*., 2003).

1.7.2.2 Expression systems based on insect cells

Insect cell expression systems can be used as alternate systems for mammalian expression system to overproduce mammalian proteins, because of the ability of post-translational modifications. The most used insect cell expression system is based on the baculovirus. *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is a double

stranded DNA virus surrounded by a lipid membrane which infects a very narrow range of insect hosts (Fraser *et al*., 1992). This virus is known to infect only members of the Lepidoptera and Hymenoptera class of insects and to be safe for other insects and humans. Baculovirus production occurs in a biphasic lifecycle. In first phase virus is produced as nucleocapsids in the nucleus and buds out of the plasma membrane. In the second phase the virus particles are occluded in the polyhedrin protein to form a crystal polyhedra, rendering them an environmentally stable structure to be carried on to other insects in the field. The non-occluded form is the one used in cell culture. The polyhedrin gene is replaced by the gene of interest to be expressed. So this gene is under the control of a strong polyhedrin promoter which gives rise to production of polyhedrin protein, upto 30% of the cell protein during the second phase of life cycle.

In the baculovirus expression system, two vectors are used, a non-modifiable viral genome and a transfer vector with the foreign gene and being capable of homologous recombination with the viral genome. These two components recombine in the cell to form a functional baculovirus genome. A relatively easier technique of recombination was developed as Bac-to-Bac® by Invitrogen. A plasmid pFastBac™ having the expression cassette is transformed into the *E. coli* strain DH10Bac™ containing parent baculovirus bacmid [bMON14272 (136Kb)]. Recombination occurs in *E. coli* and this bacmid is isolated and transfected into the insect cells (Bac-to-Bac, Invitrogen). Since the foreign gene is under the control of the polyhedrin promoter the recombinant protein will be produced when the polyhedrin promoter is switched on. The polyhedrin promoter is a late phase promoter which normally starts after 48 hr of infection. Alternately the p10 promoter is used which is an early phase promoter. Commonly used cell lines for the insect cells are Sf9, Sf21 from *Spodoptera frugiperda* ovary and High Five from *Tricoplusia ni* ovary. Another way of protein production using insect cells is based on the stable integration of the foreign gene into the insect cell genome. *Drosophila melanogaster* cell expression uses this kind of stable expression without any virus mediation. Stable expression is possible in cell lines from the moth as well. In this system the early phase promoter, *OpIE2* from *Orgyia pseudotsugata* is used. The production levels are lower than the baculovirus expression system.

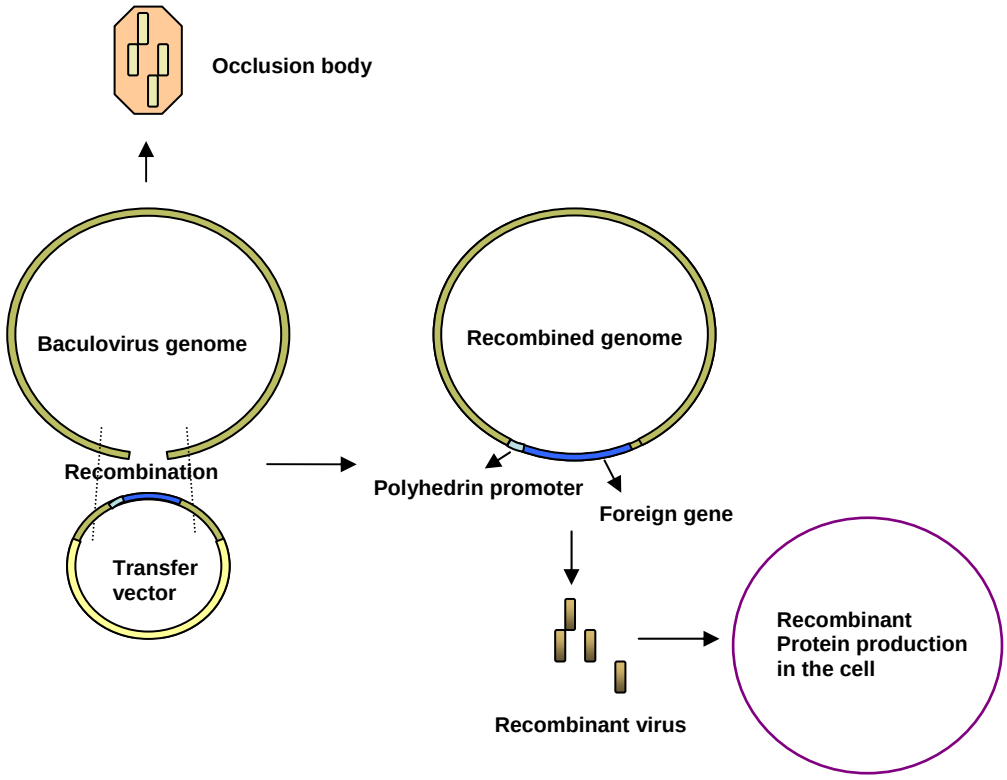


Fig 1.15: Baculovirus mediated protein production insect cells. The linear baculovirus genome and the transfer vector having recombination sites and a foreign gene under the control of polyhedrin promoter recombine in the cell. This recombined genome produces the recombinant virus and drives protein production under the control of virus promoter.

Though insect cell based expression systems are invaluable in recombinant eukaryotic protein production, they do have a set of drawbacks. The doubling rate of the cells commonly used in this system is about 18-24 hr, which makes it a slower system. Another practical problem is that the protein production is not good when the infection is done at a high cell density. Scaling up is not as easy as in *E. coli* or yeasts. Further the media used is complex and expensive compared to the earlier discussed expression systems. Several GPCRs were successfully expressed in higher yields in insect cells. Though some of them do not show high affinity binding. Cholesterol is less abundant in the insect cell membranes which might alter the function of the GPCRs produced. One advantage of the insect cell expression system is that some human GPCRs have been shown to couple to the endogenous G proteins, which increases the high affinity binding state of the receptor.

1.7.2.3 Expression systems based on mammalian cells

The mammalian expression system is the most natural way of producing a functional recombinant mammalian protein. The advantage of the mammalian expression system is that all the post-translational modifications are provided by the cell line. The functional analysis of the recombinant protein *in vivo* is possible to a greater extent than in the insect cells because of the presence of the effector molecules. There are two major approaches for heterologous expression of recombinant proteins in mammalian cells: transient and stable expression. Several virus types like vaccinia virus (Moss *et al*., 1991), Semliki Forest virus (Liljestrom *et al*., 1991) are used for transient expression. Stable expression can be achieved by stable integration of the foreign gene into the genome or by vectors capable of episomal replication. The biggest disadvantage of the system is the high costs of the media components and longer time scales. The β_2-adrenergic receptor was expressed to 200 pmol/mg in CHO cells and bovine rhodopsin upto 10 mg/l in HEK293S cells. Scaling up is difficult with mammalian cultures.

1.8 GATEWAY® cloning technology

The "Gateway" cloning technology is a novel system for cloning DNA. This system uses phage λ based site (*att*) specific recombination instead of restriction endonucleases and ligase, as in the classical way of cloning. This recombination system is used by phage λ during the switch between the lytic and lysogenic pathways. Two reactions constitute the Gateway cloning technology. The BP reaction is a recombination reaction between an expression clone (or an *att*B-flanked PCR product) and a donor vector to create an entry clone. The LR reaction is a recombination reaction between an entry clone and a destination vector to create an expression clone and a by-product.

Table 1.3: Reactions in Gateway cloning technology

Reaction	Sites involved	Enzyme mix	Product
BP reaction	*att*B x *att*P	BP Clonase Enzyme Mix	EntryClone (Plasmid)
LR reaction	*att*L x *att*R	BP Clonase Enzyme Mix	Expression Clone (Plasmid)

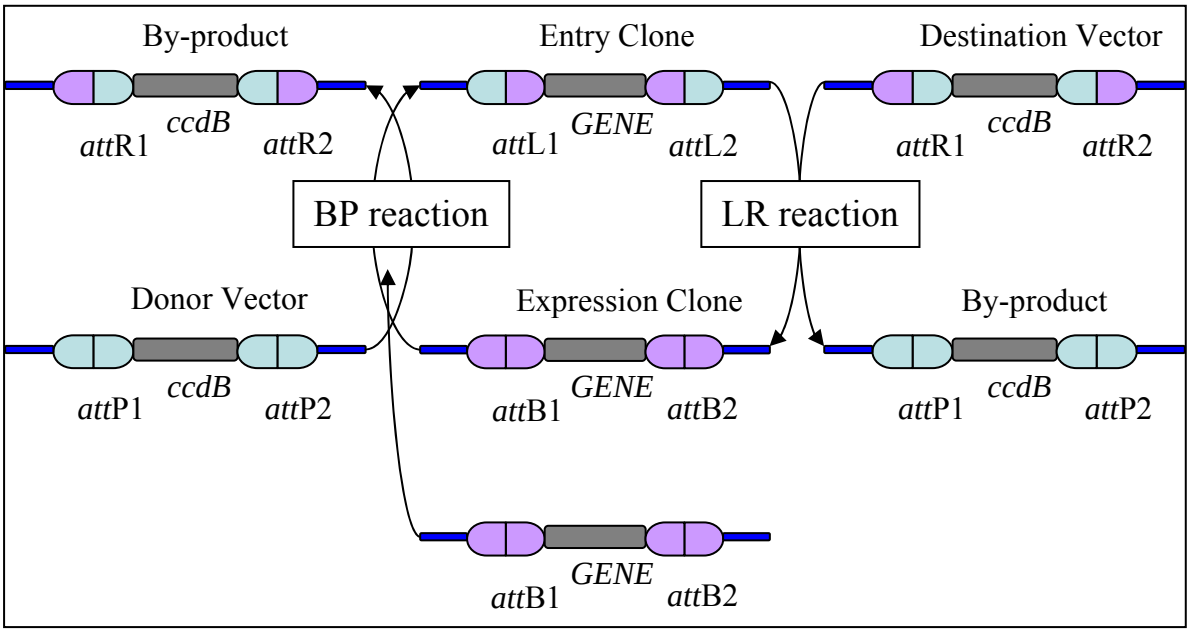


Figure 1.16: Important reactions and the products of the Gateway Cloning Technology. *att*B, *att*P, *att*L, *att*R represent different recombination sites. BP reaction is recombination of *att*B and *att*P, LR reaction is recombination of *att*L and *att*R. *ccd*B is the gene for *E. coli* cytotoxin.

The expression clone can be transformed into *E. coli* and more than 90% of the colonies obtained are positive. This high efficiency is because of the *ccd*B gene in the destination vector. Any original destination vector left in the LR reaction would cause the transformed cells to die because of the *ccd*B gene product interference with DNA gyrase. Only colonies containing expression plasmid would grow on the plate.

1.9 Fluorescent techniques

1.9.1 Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) or Förster resonance energy transfer is a energy transfer between two potentially fluorescent molecules, when they are within a distance called Förster radius. The Förster radius (R_o) is half of distance where maximum energy transfer can be seen. The energy transferred is actually nonradiative energy not the fluorescence energy. The excited molecule transfers the energy to the second molecule because of the dipole-dipole coupling. The maxium distance where FRET can be observed in the biomolecules is 100 Å. The efficiency of FRET defined as $E = 1\text{-}F'_D / F_D$

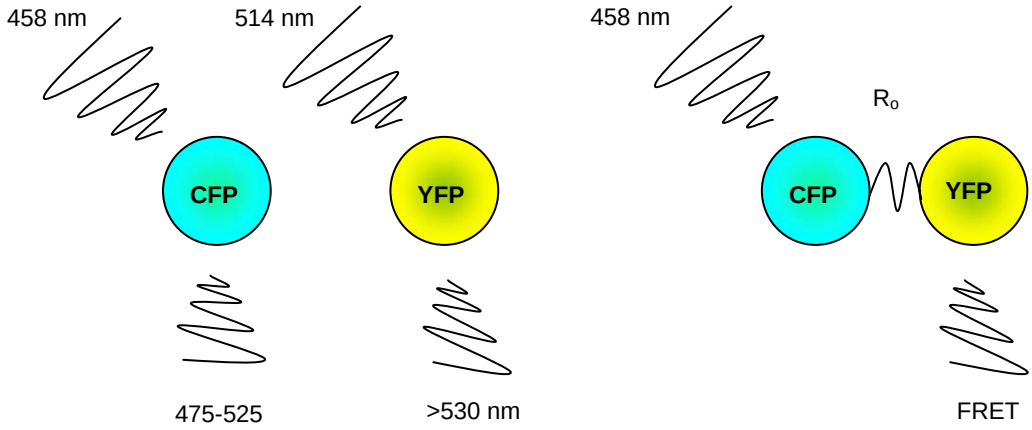


Fig 1.17: Fluorescence Resonance Energy Transfer (FRET). When both fluorescent molecules are more than 10 nm apart energy transfer doesn't occur. But below 10 nm the energy from the donor excites the acceptor molecule and results in FRET. CFP is normally excited at 458 nm and YFP at 514. The numbers below are the emission wavelengths recorded by the detector.

where F'_D and F_D are the donor fluorescence intensities with and without the presence of the acceptor. FRET occurs between two partners only when the emission spectrum of donor and the absorption spectrum of acceptor molecules overlap with other. Several GPCRs are also being studied now-a-days using the FRET technique to investigate the dimerisation behaviour and also the coupling to the G proteins or effector molecules (Nobles *et al*., 2005). Several different kinds of fluorescent molecules can be used as FRET couples. When studying protein-protein interactions *in vivo*, Green Fluorescent Protein (GFP) and its variants are commonly used. There are a series of variants which emit blue (BFP), cyan (CFP), yellow (YFP), red (DsRed) fluorescnece. These proteins can be fused by molecular biology techniques very easily.

1.9.2 Bodipy coupled GTP analogs as alternatives to GTPγS^{35}

Radioactive ligand binding assays are potentially dangerous because of the radioactive isotopes involved, even though more sensitive. But fluorescence gives a safer alternative for doing assays. Further they provide the possibility of monitoring the reactions at real time. They don't need any scintillation mixtures or special counter, not even UV optics. A normal spectrophotometer can be used to study the biological reactions. The "Bodipy" dye coupled to guanine nucleotides provide an efficient way of monitoring

GTPase activities (McEwen *et al*., 2001). The fluorescence of the Bodipy molecule is quenched by the rapid collisions between the fluorophore and guanine ring in the free form. But when the guanine nucleotide binds to the protein, the guanine ring moves away from the bodipy dye, resulting in an increase in the fluorescence. So this is a kind of dequenching mechanism. Korlach *et al.* (2004) found out that, the greater the length of the linker between the dye and nucleotide the lesser is the quenching.

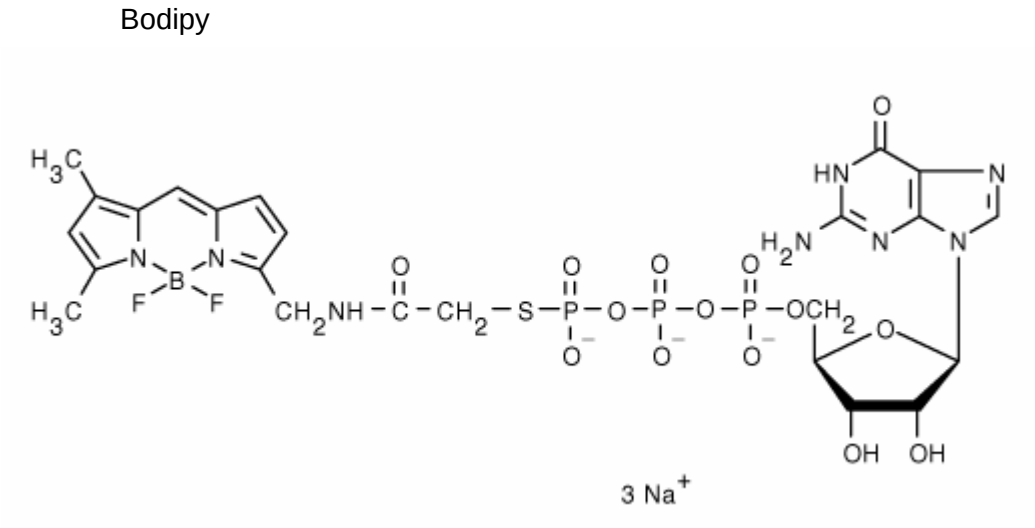


Fig 1.18: BODIPY-FL coupled to GTPγS molecule. In this molecule the guanine ring overlaps on the BODIPY fluorophore and results in quenching of the fluorescence. Once the molecule is bound to the G protein guanine ring moves away from the BODIPY resulting in increase in the fluorescence.

2. Materials and Methods..

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals used were of analytical grade and of the highest purity available. The common laboratory chemicals were purchased from Roth (Carl Roth & Co. KG, Karlsruhe, Germany), Merck (Merck KGaA, Darmstadt, Germany), Fluka/Sigma (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) (DE and Germany used alternately for Germany).

Agarose	Bethesda Research Laboratories GmbH, Neu-Isenburg, Germany
Agarose (Low melting)	FMC Bioproducts, Rockland, USA
AM251	Tocris, Ellisville, USA
AM630	Alexis Biochemicals, USA
Ammonium persulphate	Koch-Light Ltd., Haverhill, England
Ampicillin, sodium salt	Carl Roth GmbH & Co. KG, Karlsruhe
Bacto Agar	Difco Laboratories, Detroit, USA
Bacto Tryptone	Difco Laboratories, Detroit, USA
Bacto Yeast Extract	Difco Laboratories, Detroit, USA
BCIP	Biomol Feinchemikalien GmbH, Hamburg,DE
Biotin	Calbiochem, Merck KGaA, Darmstadt
Bodipy FL GTPγS	Molecular Probes, USA
BSA	Sigma-Aldrich Chemie GmbH, Deisenhofen
Bromophenol blue	Sigma-Aldrich Chemie GmbH, Deisenhofen
CHS	Sigma-Aldrich Chemie GmbH, Deisenhofen

Coomassie Brilliant Blue R-250	Serva Elektrophoresis GmbH, Heidelberg, DE
Deoxynucleotide -5'-triphosphates	Pharmacia Biotech,USA
DMSO	Sigma-Aldrich Chemie GmbH, Deisenhofen
DTT	Serva Elektrophoresis GmbH, Heidelberg, DE
EDTA	GERBU Biotechnik GmbH, Gaiberg, DE
Ethidium bromide	Bio-Rad Laboratories GmbH, Munich, DE
GDP	Sigma-Aldrich Chemie GmbH, Deisenhofen
Geniticin sufate G418	Novabiochem, Merck KGaA, Darmstadt
Glass beads (0,5mm)	Biomatik GmbH, Frankfurt, DE
GppNp	Sigma-Aldrich Chemie GmbH, Deisenhofen
GTPγS	Sigma-Aldrich Chemie GmbH, Deisenhofen
HU210	Tocris, Ellisville, USA
Kanamycin	Carl Roth GmbH & Co. KG, Karlsruhe
Lorglumide	Sigma-Aldrich Chemie GmbH, Deisenhofen
Milk powder	Nestlé, Munich, Germany
NBT	Biomol Feinchemikalien GmbH, Hamburg
Peptone	Sigma-Aldrich Chemie GmbH, Deisenhofen
PEI	Sigma-Aldrich Chemie GmbH, Deisenhofen
Rotisol30	Carl Roth GmbH & Co. KG, Karlsruhe
Rotiscint eco plus	Carl Roth GmbH & Co. KG, Karlsruhe
Silver nitrate	Sigma-Aldrich Chemie GmbH, Deisenhofen
TEMED	Koch-Light, Haverhill, England
Tunicamycin	Sigma-Aldrich Chemie GmbH, Deisenhofen
WIN 55,212-2	Tocris, Ellisville, USA
Yeast Nitrogen Base	Difco Laboratories, Detroit, USA

2.1.2 Radiolabeled chemicals

[^{3}H] CCK-8	Amersham Biosciences, UK
[^{3}H] CP-55,940	Perkin Elmer life sciences, Boston, USA
[^{3}H] SR141716A	Amersham Biosciences, UK
[^{35}H] GTPγS	Perkin Elmer life sciences

2.1.3 Detergents

CHAPS	Calbiochem, Merck KGaA, Darmstadt
Deoxycholate, Sodium salt	Sigma-Aldrich Chemie GmbH, Deisenhofen
Digitonin	Sigma-Aldrich Chemie GmbH, Deisenhofen
n-Decyl-β-D-maltoside(DM)	Glycon Biochemicals, Luckenwalde, DE
n-Dodecyl-β-D-maltoside(LM)	Glycon Biochemicals, Luckenwalde, DE
N-Dodecylphoscholine(Fos12)	Anatrace, Maumee, USA
N-Tetradecylphoscholine(Fos14)	Anatrace, Maumee, USA
N-Hexadecylphoscholine(Fos16)	Anatrace, Maumee, USA
LDAO	Anatrace, Maumee, USA
n-Octyl-β-D-glucopyranoside (OG)	Glycon Biochemicals, Luckenwalde, DE
n-Octyl-β-D-maltoside(OM)	Glycon Biochemicals, Luckenwalde, DE
SDS	Carl Roth GmbH & Co. KG, Karlsruhe

2.1.4 Protease inhibitors

Aprotinin	Biomol Feinchemikalien GmbH, Hamburg,DE
E-64	Biomol Feinchemikalien GmbH, Hamburg
Leupeptin	Biomol Feinchemikalien GmbH, Hamburg
Pepstatin	Biomol Feinchemikalien GmbH, Hamburg
PMSF	Carl Roth GmbH & Co. KG, Karlsruhe

2.1.5 Antibodies

Anti-flag M1 antibody	Sigma-Aldrich Chemie GmbH, Deisenhofen
Anti-flag M2 antibody	Sigma-Aldrich Chemie GmbH, Deisenhofen
Anti-flag M2-AP conjugate	Sigma-Aldrich Chemie GmbH, Deisenhofen
Anti-polyhistidine antibody	Sigma-Aldrich Chemie GmbH, Deisenhofen
Anti-polyhistidine-AP conjugate	Sigma-Aldrich Chemie GmbH, Deisenhofen
Streptavidin-AP	Sigma-Aldrich Chemie GmbH, Deisenhofen
Anti Gαi antibody	Calbiochem, Merck KGaA, Darmstadt
Anti Gβ antibody	Calbiochem, Merck KGaA, Darmstadt

Anti-Mouse IgG, AP conjugated	Sigma-Aldrich Chemie GmbH, Deisenhofen
Anti-Rabbit IgG, AP conjugated	Sigma-Aldrich Chemie GmbH, Deisenhofen

2.1.6 Chromatographic resins and columns

Anti-Flag™ M2 Affinity Gel	Sigma-Aldrich Chemie GmbH, Deisenhofen
ImmunoPure® Immobilized Monomeric Avidin Gel	Pierce, Rockford, USA
Ni-NTA® Agarose	Qiagen GmbH, Hilden, Germany
His-Select Agarose	Sigma-Aldrich Chemie GmbH, Deisenhofen
Profinity IMAC®	Biorad, Munich, Germany
Hi-Trap® Ni-IMAC column	Amersham Pharmacia Biotech, Freiburg, DE
Strep-Tactin®-Agarose	IBA BioTAGnology, USA
Superdex 200 PC 3.2/30	Amersham Pharmacia Biotech, Freiburg
Q-Sepharose	Sigma-Aldrich Chemie GmbH, Deisenhofen

2.1.7 Enzymes

KOD Hot start polymerase	TOYOBO, Japan
Restriction endonucleases	MBI Fermentas GmbH, St.Leon-Rot, DE New England Biolabs GmbH, Schwalbach
T4 DNA ligase	New England Biolabs GmbH, Schwalbach
Benzonase	Merck KGaA, Darmstadt, DE
EndoH	New England Biolabs GmbH, Schwalbach
PNGaseF	New England Biolabs GmbH, Schwalbach

2.1.8 *Sf9* culture media and components

TNMFH medium w/o Glutamine	CC Pro GmbH, Germany
SF 900 II medium with Glutamine	GIBCO®, Invitrogen Corporation, USA
Express Five medium	GIBCO®, Invitrogen Corporation, USA
Foetal kalf serum	PAA Laboratories GmbH, Pasching
L-Glutamine	PAA Laboratories GmbH, Pasching
Gentamycin Sulphate	Biowest, Nuaille

Vitamin B_{12}	Sigma-Aldrich Chemie GmbH, Deisenhofen
Pluronic F 68	Sigma-Aldrich Chemie GmbH, Deisenhofen

2.1.9 Kits

Qiagen Plasmid miniprep kit	Qiagen GmbH, Hilden
QIA quick Gel Extraction kit	Qiagen GmbH, Hilden
QIA quick PCR Purification Kit	Qiagen GmbH, Hilden
BCA Protein Assay Kit	Pierce, Rockford, USA
E.coli Expression Systems with Gateway Cloning Technology Kit	Invitrogen Corporation, USA

2.1.10 Buffers and Solutions

Agarose gel (1%)	1 g agarose in 100 ml 1X TAE buffer
Ampicillin Stock solution (1000x)	150 mg/ml in sterile water (-20ºC)
Alkaline Phosphatase Buffer	100 mM Tris/HCl, pH 9.5
	100 mM NaCl
	5 mM $MgCl_2$
Aprotinin (1000x)	10 mg/ml in water (-20ºC)
APS	10% w/v in water
BCIP solution	50 mg/ml in DMF (-20ºC)
Blotting buffer 5x	190 mM glycine
	50 mM Tris
Blotting buffer 1x	38 mM glycine
	10 mM Tris
	20% (v/v) methanol
Conditioner (Silver staining)	100 µl Sodium thiosulphate in 60 ml water
Coomassie solution	0.25 g Coomassie Brilliant Blue R250 in
	100 ml (45 water+45 methanol+10 acetic acid)
Destaining solution (Coomassie gels)	1 L methanol
	1 L water
	200 ml acetic acid
Developer (Silver staining)	1.2 g sodium carbonate, 25 µl formaldehyde

	25 µl sodium thiosulphate in 60 ml water
dNTP mix (10x)	8 mM (2 mM each dATP,dCTP,dGTP,dTTP)
E64 (10000x)	3.5 mg/ml 50% ethanol (-20ºC)
Gel fixer (Silver staining)	60 ml 50%(v/v) acetone
	1.5 ml 50% (w/v) trichloro acetic acid
	25 µl formaldehyde
Kanamycin stock	40 mg/ml in water (-20ºC)
Leupeptin(2000x)	10 mg/ml in water (-20ºC)
Loading buffer 4x (SDS PAGE)	25 mM Tris/HCl, pH 6.8
	40% (w/v) glycerol
	20% (v/v) β-Me
	8% (w/v) SDS
	0.004% (w/v) bromophenol blue
Loading buffer 20x (Agarose gels)	50 mM Tris/HCl, pH 7.4
	5 mM EDTA
	50% (v/v) glycerol
	0.5% (w/v) bromophenol blue
	0.5% (w/v) xylene cyanol
NBT solution	50 mg NBT/ml in 70% DMF in water (-20ºC)
PepstatinA 5000x	10 mg/ml in methanol (-20ºC)
PMSF	200 mM in isopropanol
Radioligand binding buffer	20 mM Tris/HCl pH 7.4
	100 mM NaCl
	5 mM $MgCl_2$
	1 mM EDTA
	1% BSA
Staining solution (Silver staining)	0.8 ml 20% (w/v) silver nitrate
	0.6 ml formaldehyde (37%) in 60 ml water
SDS gel running buffer (10x)	500 mM Tris
	1.92 M glycine
	1% (w/v) SDS
TAE buffer (50x) pH 7.4	2 M Tris
	1 M acetic acid
	50 mM EDTA

TBS buffer	50 mM Tris/HCl, pH 7.4 150 mM NaCl

2.1.11 *E. coli* media recipes

LB medium for *E. coli*	1% (w/v) tryptone 0.5% (w/v) yeast extract 0.5% (w/v) NaCl
LB-Agar for Plates	LB-medium with 1.5% agar

2.1.12 *P. pastoris* media recipes

The stock solutions of YNB, biotin, phosphate buffer were prepared according to the recipes given in the manual (Multi-copy *Pichia* Expression Kit) from Invitrogen life technologies. The procedure to make the media is also from the manual.

BMGY/BMMY medium (Buffered Glycerol/Methanol complex)	1% yeast extract 2% peptone 100 mM potassium phosphate, pH6.0 1.34% YNB 4x 10^{-5} % biotin 1% glycerol / 0.5% methanol
MD plates (Minimal Dextrose)	1.34% yeast nitrogen base 4x 10^{-5} biotin 2% dextrose 1.5% agar
YPD medium (Yeast extract Peptone Dextrose)	1% yeast extract 2% peptone 2% dextrose 1.5% agar (for YPD plates)
YPD Geneticin Plates	0.25-1.0 mg/ml YPD-agar

2.1.13 *Sf9* media recipe

TNMFH medium	4 mM L-glutamine
	5% (v/v) FCS
	50 µg/ml gentamycin
	10 µg/L Vit B12
Plaque assay plates	TNMFH complete medium
	2% (w/v) low melting agarose
	X-gal 200 µg/ml

2.1.14 Instruments

Confocal microscope LSM 510	Carl Zeiss AG, Germany
Electroporation device	Biorad, Munich
Fluorescence microscope	Carl Zeiss AG, Germany
Gel documentation system	Biorad, Munich
Light Microscope	Olympus Inc., Japan
Luminescence photometer LS50B	PerkinElmer LAS (Germany) GmbH, Rodgau
Optima LE-80K UC	Beckman-Coulter, Fullerton, CA, USA
Radioactivity counter	PerkinElmer LAS (Germany) GmbH, Rodgau
Shakers	Infors AG, Switzerland
Sigma 3K 12 tabletop centrifuge	Sigma Laborzentrifugen GmbH, Osterode
Sorvall RC-5B	Sorvall, Bad Homburg
Spectrophotometer	Thermo Spectronic, UK
Tabletop Eppendorf 5415D	Eppendorf GmbH, Hamburg
Tabletop Ultracentrifuge TL 100	Beckman Instruments Inc., Palo Alto, USA
Thermomixer 5436	Eppendorf GmbH, Hamburg
Vortexer	Bender & Hobein AG, Zurich, Switzerland

2.1.15 Consumables

15ml/50 ml culture tubes	Greiner bio-one
Disposable pipets	Sarstedt, Nümbrecht, Germany
Glass bottles	Schott AG, UK

Microfuge tubes	Eppendorf GmbH, Hamburg, Germany
Pipette tips	Sarstedt, Nümbrecht, Germany
Syringe filters	Sarstedt, Nümbrecht, Germany
Tissue culture flasks	Nunc GmbH & Co. KG, Wiesbaden, DE

2.2 Methods

2.2.1 Working with *Escherichia coli*

2.2.1.1 Culturing *E. coli*

A single colony on the freshly transformed or streaked LB-agar plate was used to inoculate 5 ml LB medium with appropriate antibiotic. The culture was grown overnight at 37ºC and 220 rpm. This culture was used for further experiments like plasmid DNA isolation or as seed culture for expression of the transformed gene.

2.2.1.2 *E. coli* competent cell preparation and transformation

Tranformation Buffer (TB):
15 mM $CaCl_2$
55 mM $MnCl_2$
250 mM KCl
10 mM PIPES-Na, pH 6.7

For the high efficiency competent cells preparation, a single colony of *E. coli* was grown overnight in 2 ml LB medium. This was used to seed 100 ml LB medium containing 10 mM $MgCl_2$ and grown at 18ºC, shaking at 100 rpm. When the OD_{600} was 0.1, the culture was cooled on ice for 10 min. Cells were pelleted using a centrifuge and resuspended in 1/3 culture volume (33 ml) of TB. The suspension was pelleted again and resuspended in 8 ml of TB. 600 µL of DMSO was added. The suspension was aliquoted and flash frozen in liquid nitrogen before storing at -80ºC.

To transform plasmid DNA, an aliquot of the competent cells was thawed on ice. Plasmid DNA (100-1,000 ng) was added and incubated on ice for 20 min. The cells were given a heat shock at 42ºC for 2 min. The transformed mixture was spread evenly on a LB-agar plate with an appropriate antibiotic. The plate was incubated over night at 37ºC. A single colony was used for any further experiments done.

2.2.1.3 Gene amplification by PCR

To amplify the genes from cDNA, Hot start KOD or *Pfu* polymerase was used. The amplification conditions were according to the supplier's manual. To incorporate a foreign gene inside another gene (Gi-CFP), a typical overlap PCR was done (see Appendix).

2.2.1.4 Cloning of *E. coli* expression vectors

Introducing a foreign gene into a vector was done in one of two ways for the *E. coli* expression system. In the classical method, the vector and the PCR product were digested with the compatible restriction endonucleases according to the supplier's instructions. The vector and the PCR product were purified using the gel extraction kit/ PCR purification kit. The ligation was done using T4 DNA ligase for 2 hr at 24ºC or overnight at 15ºC. Ligation reaction was stopped by heat inactivation of the enzyme at 65ºC for 15 min. 6 µL of the ligation mix was used to transform the competent *E. coli* cells. The cells were plated on LB-agar plate and incubated overnight. Positive colonies were identified by isolation of plasmid DNA (Plasmid isolation kit) and restriction digestion. Alternatively the GATEWAY cloning technology from Invitrogen was used. The reactions of this cloning technology were performed according to the protocols given in the manual. The entry vector pENTR11 and destination vector pDEST14 were used for this work. pDEST uses the *T7* promoter. Tags were introduced into the genes by PCR. Oligonucleotides used for the PCR amplification of the G protein subunits contained the following sequences:

Forward(*att*B1): 5' GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC GAA GGA GAT AGA ACC ATG GTG CAT CAT CAT CAT CAT CAT XXX XXX XXX 3'

Reverse(*att*B2): 5' GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TCA XXX XXX XXX 3'

2.2.1.5 Expression screening in *E. coli*

For the screening of protein expression in *E. coli,* expression hosts like BL21(DE3), BL21-CodonPlus RP/RIL, Rosetta(DE3), Rosettagami(DE3), C43(DE3) were used. The plasmid with the gene of interest was transformed into the competent cells of expression the strain. A single colony was picked from the LB-amp plate for a 2 ml overnight seed culture. 5 ml cultures were inoculated with 1% of the overnight culture and incubated at 37ºC. At an OD_{600} of 0.5-0.6, expression was induced by addition of IPTG at concentrations of 50 and 200 µM. The cultures were incubated at 30ºC for different intervals of time. 1 ml culture was aspired at regular intervals from all the culture tubes. This aliquot was pelleted and stored at -20ºC until use. The pellets were resuspended in 200 µl of buffer (20 mM Tris/HCl, 100 mM NaCl). The suspension was heated on a thermo block at 95ºC for 10 min, to lyse the cells. The debris was pelleted by centrifugation and the supernatant was used either for SDS-PAGE or for 96 well dot blot.

2.2.2 Working with *Pichia pastoris*

2.2.2.1 Maintaining yeast culture

Pichia pastoris was maintained in YPD medium. A single colony from a freshly streaked YPD plate was inoculated into YPD medium and incubated overnight at 30°C.

2.2.2.2 *P. pastoris* transformation

The transformation in *P. pastoris* was done by electroporation. A single colony of the strain SMD1163/SMD1168 was inoculated in 2 ml YPD medium and grown overnight. A 100ml culture was inoculated from the overnight culture at an OD_{600} equal to 0.1. The cells were grown to a OD_{600} of 1.5. The culture was cooled on ice for 30 min. The cells were pelleted by centrifugation and resuspened in 100 ml of autoclaved water. This washing step was to remove excess medium and buffer components. The suspension was pelleted again and resuspened in 50 ml water. One more washing was done using 2 ml of 1 M sorbitol. Finally the pellet was resuspended in 200 µl of 1M sorbitol. This suspension was used for electroporation.

The supercoiled plasmid was linearised using one of the suitable enzymes (mostly *Pme*I) and purified using the Qia-quick PCR purification kit. 80 µl of cell suspension was mixed with 10-20 µl (10 µg) of linearised DNA and incubated on ice for 5 min. The suspension was added to a 2 mm electroporation cuvette. Electroporation was done using a Bio-rad electroporation device with 600 mA current and 1.5 kV voltage at 25 µF capacitance. A short pulse was given and the time constant was monitored (11-12.5) to assess the successful transformation. 1 ml of 1 M Sorbitol was added to the transformed cells and 200 µl of this solution was plated onto an MD plate. The plates were wrapped with parafilm and incubated at 30°C till prominent colonies appeared.

2.2.2.3 96 colony screening for selecting high expression clone

The colonies obtained on the MD plate after transformation are *His*$^+$, which means that they are right recombinants with a stable integration of the gene of interest. Since in *P. pastoris* the expression levels depend very much on the number of integrated genes, there is a need to screen maximum possible number of colonies. In this work a rapid expression screening technique was used alternately for screening based on antibiotic resistance.

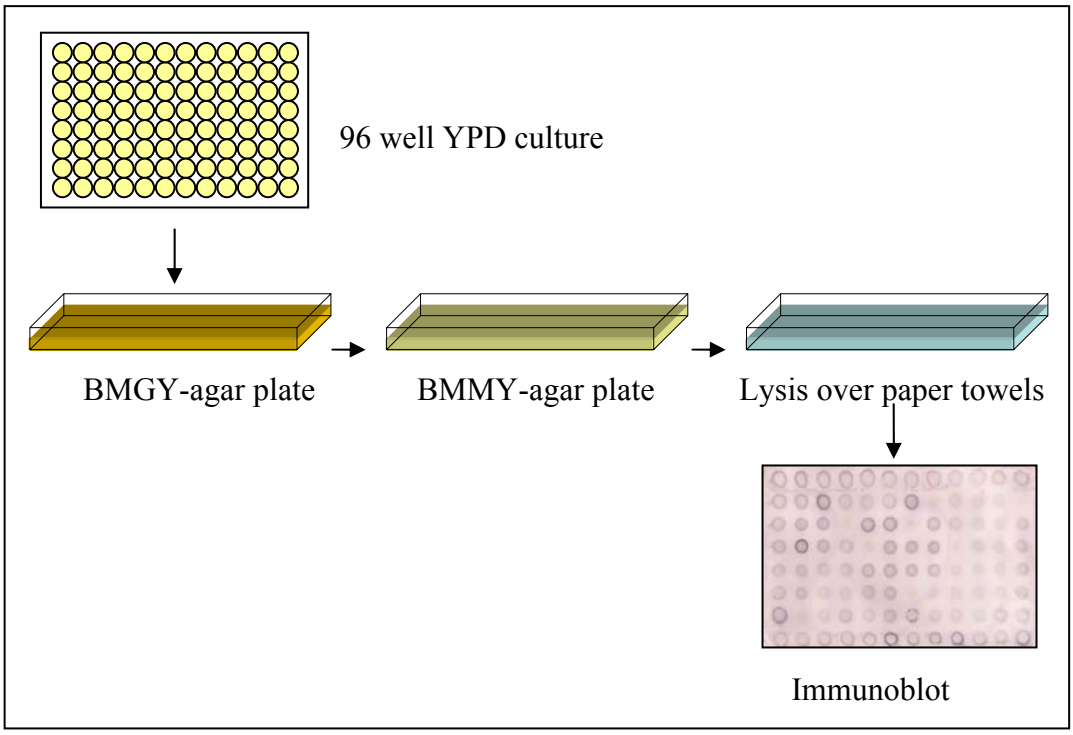


Figure 2.1: Steps in the rapid expression screening. Colonies were grown for about 2 days on BMGY plates and 1-2 days on BMMY plates. Lysis was done at 65°C for 3 hr. Immunoblot was done using the epitopes on the protein of interest.

96 colonies were picked from a fresh MD plate and inoculated in a 96 well plate containing 200 µl of YPD per well. The plate was incubated overnight. This culture was replica copied onto a nitrocellulose paper, placed over BMGY-agar in a 1 well rectangular dish. The plate was incubated in a humid chamber in a 30°C incubator. When the colony grew to about 3 mm in diameter on the nitrocellulose membrane (NC), the paper was removed from the BMGY plate and placed on a BMMY plate. The plate was incubated again for 24-48 hr.

After the incubation, colonies on the NC paper were lysed using the lysis buffer (50 mM Tris/HCl, 100 mM NaCl, 10 mM $MgCl_2$, 50 mM β-Me, 6 M urea, 2% SDS). The NC membrane with colonies was placed over paper towels presoaked with the lysis buffer and incubated at 65°C for 3 hr. The dried colony debris was washed with water properly using a wash bottle. This washed paper was used for immunostaining. The colony which gave the brightest dot was selected and stored as glycerol stocks (Fig 2.1).

2.2.2.4 Preparation of *P. pastoris* cell membranes

For the large scale purification of a soluble protein or to prepare the membranes of *P. pastoris* the cells were broken using glass beads. The cells were pelleted and the pellet was resuspended in breaking buffer, pH 7.4 (25 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$, PMSF). Typical 1 L shaking flask culture pellet was re-suspended in 50 ml buffer and chilled on ice. An equal volume of 0.5 mm glass beads was added to the suspension in a beaker. The suspension was stirred at 1000 rpm for 15 min using a homogenizer fitted with a metal rod with spikes at the bottom. The suspension was filtered through a sieve funnel, to remove the glass beads. The cell suspension was centrifuged at 3000 rpm for 10 min to remove the unbroken cells and cell debris. The supernatant was centrifuged at 150,000 g in a ultracentrifuge (UC), to pellet the cell membrane. For soluble proteins, the supernatant after UC was used for further steps of purification. For membrane proteins, the pellet from UC was homogenized using a potter, in a resuspension buffer (breaking buffer +10% (v/v) glycerol). The homogenized membrane was flash frozen and stored at -80°C, until further usage.

2.2.2.5 Storage of positive clones of *P.pastoris*

Since the transformants of *P. pastoris* possess stable integration of the gene, it is very important to store the clones properly. The positive clones were grown to an OD_{600} of 0.5 in YPD medium. Glycerol was added to 30% (v/v) of culture volume. The culture was divided into 1 ml aliquots, flash frozen in liquid nitrogen and stored in -80°C refrigerator.

2.2.3 Working with insect cells

2.2.3.1 Maintaining insect cell cultures

The *Spodoptera frugiperda* pupal ovarian cell line *Sf9* was maintained in complete TNMFH medium. The cell line was maintained in tissue culture flasks as well as in suspension. For shaking suspension cultures 0.1% (w/v) Pluronic F-68 was included in the medium to reduce the shearing of cells. The cells were grown in 27°C incubators. The suspension culture was grown in a shaker at 150 rpm, in a square bottle or a conical flask.

2.2.3.2 Baculovirus DNA transfection of insect cells

Transfection of the virus DNA (Baculo Gold) and the pVL expression plasmid DNA into the *Sf9* cells was done using Cellfectin reagent. A transfection mix was made with the following components.

0.4 µg pVL plasmid DNA

0.1 µg Baculo Gold (4µl)

30 µl transfection reagent

were mixed and the volume was adjusted to 50 µl with sterile water. The transfection mix was incubated for 15 min at room temperature. Meanwhile 1.28 x 10^6 cells were added to each well of a 6 well culture plate. The cells were washed 3 times with medium without FCS. 1 ml medium without FCS was added to the transfection mix and then added to the cells. The plates were incubated at room temperature for 1 hr. The solution was removed from the well and 2 ml fresh medium with FCS was added to the cells. The plates were then incubated at 27ºC for one week, to produce mother stock virus. After one week the medium was centrifuged in a 2 ml Eppendorf tube. The clear supernatant was stored at 4ºC.

2.2.3.3 Plaque assay

Plaque assay was done to select the clones producing the protein of interest. $2x10^6$ cells were added to each 5 cm round Petri-dish. Cells were allowed to attach to the bottom. They were washed thrice with medium without FCS. The mother stock virus was diluted in the order of 10^{-1} to 10^{-5}. These diluted virus samples were added to the cells in the Petri-dish. The cells were infected for 1 hr. The supernatant was removed and 4ml of medium with agarose (1:1 mixture of 3% (w/v) LM agarose and TNMFH medium with 10% (v/v) FCS) warmed to 37ºC was added over the cells. The medium was allowed to solidify and then incubated at 27ºC for 4-6 days.

After 6 days when the plaques could be seen, 2 ml of medium with agarose containing 200 µg/ml X-gal was added. The plates were incubated for 24 hr. The white plaques were picked using a pipette and added to the cells freshly added to a 6 well plate. 5 plaques were picked for each construct. The plates infected with the plaque virus were incubated for 1 week to produce the virus for the clone. The virus containing medium was collected after one week and stored at 4ºC.

2.2.3.4 Determining the virus titre by endpoint dilution assay

The endpoint dilution assay (Summers & Smith, 1987) was done in a 96 well microtitre plate. $1x10^5$ cells were added to each well in the plate. Virus was diluted typically starting from 10^{-2} to 10^{-10}. 10 µl of each dilution was added to each well of a row (of 12 wells) in the plate. 8 dilutions were typically used, one for a row. The plates were incubated for 1 week. Then each well was scored for infection. When at least a single cell was infected in the well, it was scored positive and negative otherwise. Using this score the percentage of infection was determined for each dilution. This percentage was used to determine proportionate distance (PD) between the infection dilutions.

PD= (a-50)/(a-b)

a= nearest percentage of infection above 50%

b=nearest percentage of infection below 50%

Using the PD a factor called Tissue culture infection density at 50% infection ($TCID_{50}$) was calculated.

$\log TCID_{50}$=(dilution of a - PD)

1/log $TCID_{50}$ gives the titre of the virus per 10 µl virus used.

Plaque forming units (Pfu) were determined from the titre.

Pfu/ml=titre/ml x 0.69

Pfu was used as a standard unit to infect the cells at different MOI (multiplicity of infection).

2.2.3.5 Expression screening

Small suspension cultures were grown, to screen the conditions for a good expression of the protein. 1 MOI corresponds to an infection with virus which has equal Pfu as there are cells in the well. 1, 5, 10 MOI were used to determine the best expression levels of the protein. Different infection times were also investigated to get the best expression level. The cells from the small suspension cultures were used to check the expression of the protein.

$1x10^5$ cells were collected in a centrifuge tube and lysed using 1% SDS in a buffer (50 mM Tris/HCl) containing Benzonase and a cocktail of protease inhibitors. The tubes were incubated for 30 min on ice to allow proper lysis and digestion of DNA by Benzonase. A high speed spin was given to remove any cell debris. The supernatant was used to run a SDS-PAGE followed by immunoblotting. For large scale expressions, cells were grown first to a density of $2x10^6$ cells/ml. Cells were pelleted at 2,000 rpm in sterile centrifuge tubes. The pellet was resuspended in fresh medium to the same density and then infected with virus.

2.2.3.6 Preparation of cell membranes

Insect cells were lysed using a Parr-bomb. The cells were resuspended in a buffer containing 10% (w/v) saccharose and a cocktail of protease inhibitors (pepstatin, aprotinin, leupeptin, E64, PMSF). The cell suspension was stirred slowly under a pressure of 500 psi in the Parr-bomb for 1 hour. The pressure was released slowly and the collected suspension was spun at 3,000 rpm for 10 min to separate unbroken cells. The supernatant was ultracentrifuged at 150,000 g for 1 hour. The pellet was homogenized in resuspension buffer containing 10% glycerol. The membrane was flash frozen and stored at -80ºC.

2.2.4 Protein detection and staining

2.2.4.1 SDS-polyacrylamide gel electrophoresis

SDS-PAGE was routinely used for identifying the protein from crude samples and to assess the purity of the protein preparation. 12% separating gels were prepared (according to the protocols by Maniatis) in ready to use cassettes from Invitrogen. Samples were prepared using a 2X sample buffer. But when the samples were membrane proteins heating at 95ºC was omitted, to avoid aggregation of the membrane protein. Electrophoresis was done in a vertical unit (X Cell Sure lock) from Invitrogen. The gels were run in gel running buffer at a constant current of 25 mA for each gel. These gels were stained by Coomassie or silver or used for immunoblotting.

Separating gel:	Water	13.2 ml	Stacking gel:	10.2 ml
(for 40 ml)	Rotisol	16.0 ml	(for 15 ml)	2.5 ml
	Buffer, pH8.8	10.0 ml	Buffer pH 6.8	1.8 ml
	APS	0.4 ml		0.15 ml
	SDS (10%)	0.4 ml		0.15 ml
	TEMED	0.016 ml		0.015 ml

2.2.4.2 Coomassie staining of SDS-PAGE gel

The gel after electrophoresis was stained using Coomassie staining solution with slow shaking for 1 hr-overnight. The staining solution was removed and the gel was destained using a destaining solution, in a closed container. The container was closed to avoid excess evaporation of acetic acid and methanol. Destaining was done till the background was clear. The gel was dried in a gel drier at 80ºC and documented.

2.2.4.3 Silver staining

Silver staining of a SDS-PAGE gel was done in a glass Petri-dish. The gel was fixed in 60 ml fixer solution for 5 min and washed afterwards with Millipore water for 5 min. The gel was rinsed with water again 5 times for 3 sec to remove any fixer solution and 60 ml 50% acetone was added. After 5 min the acetone was removed and 60 ml of conditioner

was added and incubated for 1 min. The gel was washed properly with water for 5 times. Then the gel was kept in the staining solution for 8 min. Staining solution was removed and washed with excess water to remove silver ions. The developer was added to the gel and once the protein bands were visualized, acetic acid was added to stop the reaction. The gel was rinsed in water and dried for documentation.

2.2.4.4 Immunoblotting

Electric currrent was used to transfer the proteins from the gel onto a PVDF membrane, in a semi-dry method. A PVDF membrane similar in size to the gel was activated with methanol for 5sec and then transferred to transfer buffer. Filter paper was cut in dimensions similar to the gel and soaked in transfer buffer. Three soaked papers were kept on the horizontal blotting apparatus. The gel was placed on paper towels. PVDF membrane was kept on top of the gel. Three more paper towels were placed on top to make a sandwich of gel and PVDF membrane. This sandwich was slowly rolled over using a glass pipette, to remove air bubbles between the layers. The upper graphite electrode plate was placed and electroblotting was done at a constant current of 50 mA for 2 hr.

After the gel was electroblotted, the PVDF membrane was placed in a 5% solution of fatty acid free milk powder for blocking. After 20 min the membrane was washed using TBS and it was incubated for 1 hr with the antibody specific (primary) to the epitope on the protein of interest. If the antibody is not conjugated to an enzyme(AP), membrane was washed with TBS and then incubated with the secondary antibody conjugated to AP. After the antibody treatment the gel was washed thoroughly with TBS and developed using the substrate solution (66 µl NBT+ 33 µl BCIP in 10 ml AP buffer). Once the bands were visualized the membrane was washed with excess water to stop the reaction. For immunoblotting done in a dot-blot technique the membrane onto which the protein samples are blotted was directly blocked with milk powder and treated with antibody solutions in the same way as done for the electroblotting.

2.2.5 Radiolabeled ligand binding assay

For radiolabeled ligand binding assay of a GPCR, the cell membrane was incubated with different concentrations of the radiolabeled ligand ([^{3}H] SR141716A, [^{3}H] CP-

55,940) . For each concentration the assay with the radio ligand was done in triplicate and duplets for the controls containing cold ligand (3 mM WIN 55-212,2 for CB1 and CB2 receptors) in the binding buffer to a final volume of 250 µl. The reactions were incubated at 30ºC in a water bath for 1 hr. They were then filtered through glass fiber filtermats (GF/B Whatmann) in a 12 slot or 24 slot filtering apparatus using a vacuum pump. The glass fiber filters were washed with the same binding buffer at 30ºC, three times 4 ml each. Glass fiber filters were removed from the filtering apparatus and put into a scintillation vial. 4.5 ml of scintillation fluid was added to each vial. The vials were vortexed briefly and the radioactive counts were measured in a β-counter.

The GTPγS^{35} binding assay was done in a similar filter based binding assay. The membranes containing the G proteins were incubated with 50 µM GDP prior to the activation of the receptors. The CB1 receptor was activated using 1 µM WIN 55-212,2 and inactivated using AM251. The membranes were incubated with ligands at 30°C for 1 hr and the reaction mixture was filtered over GF/C filters. The filters were washed with buffer and radioactivity was measured as mentioned above.

2.2.6 Fluorescent GTPγS binding assay

Bodipy FL GTPγS was used as an alternate to the radioactive GTPγS^{35}. Bodipy FL GTPγS binding was done in a buffer containing 20 mM Tris/HCl, 100 mM NaCl, 10 mM $MgCl_2$ and 50 µM GDP. The fluorescent ligand was used at a concentration of 500 nM. The binding was monitored in a time scale program in a luminescence spectrophotometer. In the measurements of GPCR and G proteins interactions, concentrations of 20 nM and 100 nM were used respectively.

2.2.7 Protein purification

2.2.7.1 Immobilized metal affinity chromatography

Ni-NTA agarose and His-Select were used for IMAC. For purification of G protein α subunits, a large scale culture (1 L) was centrifuged and the cell pellet was resuspended in 20 ml of buffer A (20 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$, PMSF). The cells were lysed using lysozyme at 0.5 mg/ml of suspension. The suspension was incubated for 30 min

on ice and then flash frozen in liquid nitrogen. The frozen suspension was thawed slowly. After thawing, the suspension was sonicated with 3 pulses of one minute each to shear the DNA. The suspension was centrifuged at 135,000 g and the supernatant was loaded to the Ni-NTA agarose column fixed to a peristaltic pump. The cell lysate was loaded at 0.5 ml/min flow rate.

The column was first washed with 10 column volumes of buffer A containing 50 μM GDP. Second washing was done with 10 column volumes of buffer B (buffer A containing 500 mM NaCl and GDP). Third wash was with 20 column volumes buffer C (buffer A containing 30 mM imidazole and GDP). Protein was eluted with elution buffer (buffer A containing 250 mM imidazole and GDP). After elution, the eluate was concentrated and diluted several times with buffer A in a Centricon (Viva-spin), to remove imidazole.

Membrane proteins were first solubilized using a suitable detergent and centrifuged at 150,000 g for 1 hr. The supernatant or the solubilizate was loaded onto the column. To facilitate efficient binding of membrane protein, the matrix and the solubilizate were incubated under slow rocking in a cold room for 1 hr and then loaded into the column. The washing steps were similar except that a detergent was always present in all the buffers at 2 times CMC. (The buffers didn't contain GDP).

2.2.7.2 Ion-exchange chromatography

For G protein purification IMAC was followed by ion-exchange chromatography. The eluate from the Ni column was concentrated using a Centricon and diluted with a buffer containing no NaCl. This protein solution was loaded to the Q-Sepharose matrix in the column at a flow rate of 1ml/min. After loading, the column was washed with 5 column volumes of 50 mM NaCl to remove any unbound proteins. Then a gradient of 50 to 300 mM NaCl in 60 ml was applied using the High-Flow automated purification system. Fractions of 2 ml were collected and analysed by SDS-PAGE for the presence of protein. The fractions containing protein were pooled, concentrated and strored in a -80ºC refrigerator. The column was washed with 500 mM NaCl and then with water and stored in 20% ethanol.

2.2.7.3 Streptavidin affinity chromatography

Proteins fused to the biotinylation domains from *P. shermanii* were purified using a monomeric avidin based matrix. Irreversible binding sites of the monomeric avidin matrix were blocked by biotin prior to loading the protein sample. The matrix was blocked with 5 column volumes of 2 mM biotin containing buffer. Then it was washed with 5 column volumes of 0.1 M glycine (pH 2.8), to remove reversibly bound biotin. The column was equilibrated with purification buffer (20 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA). Protein sample was incubated with the matrix for 2 hr at 4ºC. The suspension was loaded onto the column and washed with 10 column volumes of equilibration buffer. The protein was eluted using 10 mM biotin in buffer. The column was washed with glycine to remove biotin then with equilibration buffer to reuse the matrix.

To purify proteins fused to Strep-tag II, a modified streptavidin, Strep-Tactin was used. Blocking of irreversible binding sites was done with biotin. To remove reversibly bound biotin the column was washed with a 10 mM solution of HABA (4-hydroxyazobenzene 2-carboxylic Acid). The column was then equilibrated with purification buffer. The protein sample was incubated with the matrix at 4ºC for 2 hr to facilitate better binding. The column matrix was washed with 10 column volumes of purification buffer to remove any unbound proteins. The protein was eluted using a 5 mM solution of desthiobiotin in purification buffer. Column regeneration was done using HABA solution.

2.2.7.4 Analytical gel filtration

Analytical gel filtration had been used to assess the homogeneity of the protein. A Superdex 200 column was used frequently in the SMART system (analytical purification system from Amersham). The column was equilibrated with 1.5 column volumes of purification buffer. The protein sample (50 µl), a minimum of 10 µg, was loaded onto the column. The column was run with the purification buffer and fractions of 50 µl were collected. The fractions were analysed by SDS-PAGE.

2.2.8 Co-immunoprecipitation

Co-immunoprecipitation was done to check the complexes of cannabinoid receptor and the G proteins. Membranes containing both CB1 receptor and G proteins were solubilized in different detergents. The solubilizate was centrifuged at 100,000xg for 1 hr and the supernatant was incubated with anti-flag M2 antibody-agarose. The matrix was washed with purification buffer to remove any unbound proteins. Bound proteins were eluted using SDS loading buffer. This eluate was analysed by immunoblotting for the presence of both proteins.

2.2.9 Fluorescence resonance energy transfer (FRET)

A confocal microscope (LSM 510, Zeiss) was used to observe the co-localization of the CB1 receptor and G proteins. The receptor and the Giα1-protein were fused to modified Green fluorescent proteins. The CB1 receptor was fused to YFP (Yellow Fluorescent Protein) at the C-terminus. CFP (Cyan Fluorescent Protein) was introduced into a loop between the α-helical domain and the GTPase domain, by overlap PCR (Yu and Rasenick, 2001). Cells containing both proteins were used to monitor FRET resulting from the co-localization of proteins. Fluorescence was recorded in channel mode using two channels, one for the acceptor (YFP) fluorescence and one for the donor (CFP) fluorescence. CFP was excited using a 458 nm laser and the fluorescence through a band pass filter of 475-525 nm was recorded. YFP was excited using a 514 nm laser and emission was recorded from a long pass filter of 530 nm. Bleaching of the receptor was done using 514 nm laser at 80% laser strength for 30 sec. The pre-bleach and post-bleach fluorescence of the donor protein was recorded from the first channel. The differences in the fluorescence intensities of the pre and post-bleach images were calculated and there by the % FRET signal was calculated using the formula

$$\%FRET = Dpost-Dpre \times 100/ Dpost$$

Dpost = Donor emission intensity in the post-bleach image

Dpre = Donor emission intensity in the pre-bleach image

2.2.10 3-Dimensional protein crystallization

To crystallize the proteins, crystallization kits from Hampton Research, Jena Biosciences and Nextal crystal screens were used. Crystallization of $G\alpha_q$ protein was done using Hampton Research screens. The buffer conditions of the positive hits were broadened to make a sparse matrix screen. One component of the buffer was varied keeping the other components constant. Crystallization was done in hanging drop setups. The protein sample at a concentration of 10 mg/ml was mixed (1:1) with buffer on a silanized cover slip and placed over the well with buffer. The plates were incubated at 18ºC. The crystals, after repeated washes were checked on SDS-PAGE. The diffraction pattern was checked at ID14, ESRF, Grenoble. An automated robotic system to setup nl sitting drops was also used, while screening a large number of conditions.

3. Results..

3 RESULTS

Chapter I: G protein production, purification and functional analysis

3.1 Cloning of G protein subunit genes

Cloning of a total of 35 genes coding for human G proteins was done in a high through put scale, using the GATEWAY cloning technology (see Introduction 1.8). The 35 genes included 17 Gα, 6 Gβ, and 12 Gγ subunits.

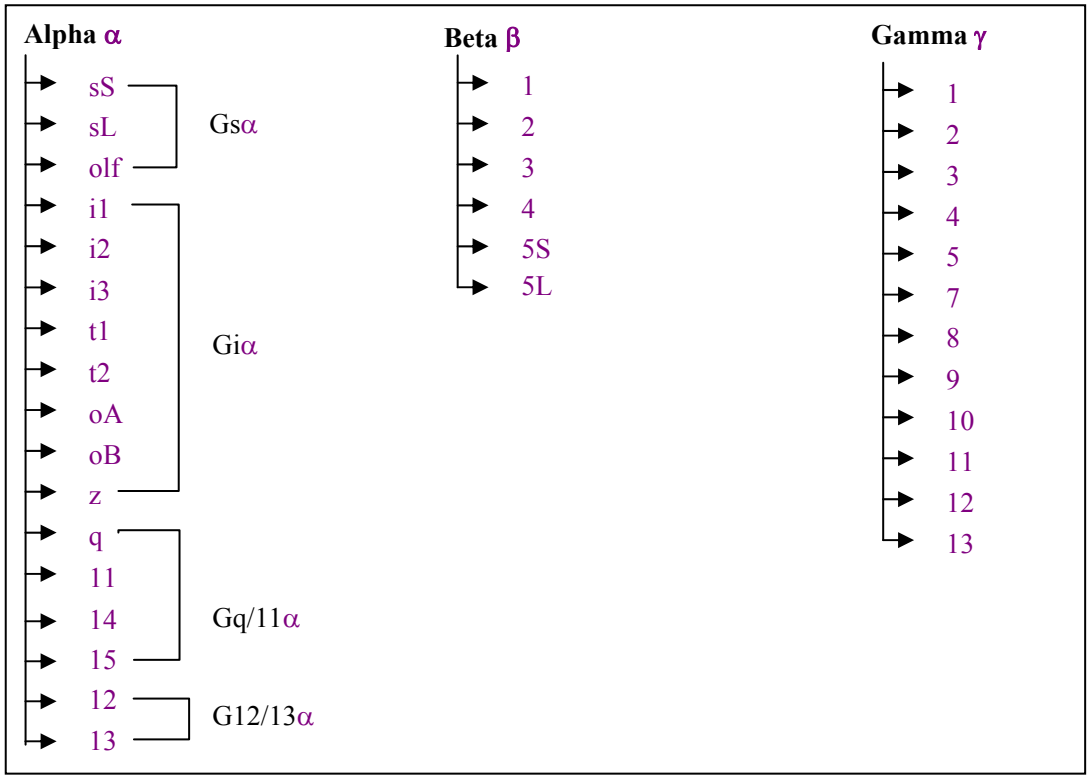


Figure 3.1: An over view on the human G protein subunits. 35 G protein subunits from human were cloned using the Gateway cloning technology into the pDEST14 vector (Appendix A1), to produce in *E. coli.*

3.2 Expression of the G protein subunit genes

Expression of G protein subunit genes was carried out in various *E. coli* expression strains. Since there were several rare amino acid codons (not used by *E. coli*) found in human G protein genes, the *E. coli* strains (materials and methods 2.2.15) used for the expression contained the tRNAs for these rare amino acid codons. The gene construct coding for an N-terminal hexahistidine tag and for the G protein gene is represented in Fig 3.2

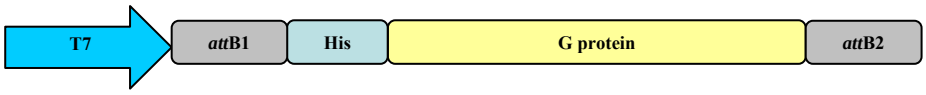


Fig 3.2: Expression construct of G protein gene in the pDEST14 vector. T7 is the T7 promoter, *att*B1 and *att*B2 are the recombination sites, and His stands for a hexahistidine tag.

3.2.1 Production of recombinant Gα subunits

The G protein α subunits were produced best among all the subunits screened (Fig: 3.3). Out of the 16 Gα subunits tried, 11 proteins could be produced in high yields (3-10 mg/liter culture).

Incubation time and the IPTG concentration had varying effects on the production of different G protein subunits. $G\alpha_{i3}$ subunit production reached a maximum after an incubation time of 6 hr, whereas other proteins were produced better with longer incubation times. For most of the produced proteins, production reached a saturation point at about 12 hr post induction, where as $G\alpha_{i1}$ showed an increase even till 20 hr. Interestingly, $G\alpha_{oB}$ and $G\alpha_{tC}$ (transducin cone type) protein production was seen at lower IPTG concentrations and absolutely no recombinant protein production at higher induction levels.

Among the Gα subunits tested, $G\alpha_{15}$ protein production was the least and $G\alpha_{i3}$ was the highest. $G\alpha_{11}$, $G\alpha_{13}$, $G\alpha_{14}$, $G\alpha_{q}$ and $G\alpha_{olf}$ subunits could not be produced in the BL21-CodonPlus strain of *E. coli*. Different expression strains were tested to produce these proteins. Rosettagami (DE3) pLysS strain gave better results for $G\alpha_{11}$, $G\alpha_{13}$, $G\alpha_{14}$, $G\alpha_{15}$ (Fig: 3.4-A) than the BL21 CodonPlus strain (assessed by dot intensity). The production level of $G\alpha_{14}$ subunit was better than others. But there was a considerable amount of protein in the insoluble fraction (may be in the form of inclusion bodies) (Fig: 3.4-B).

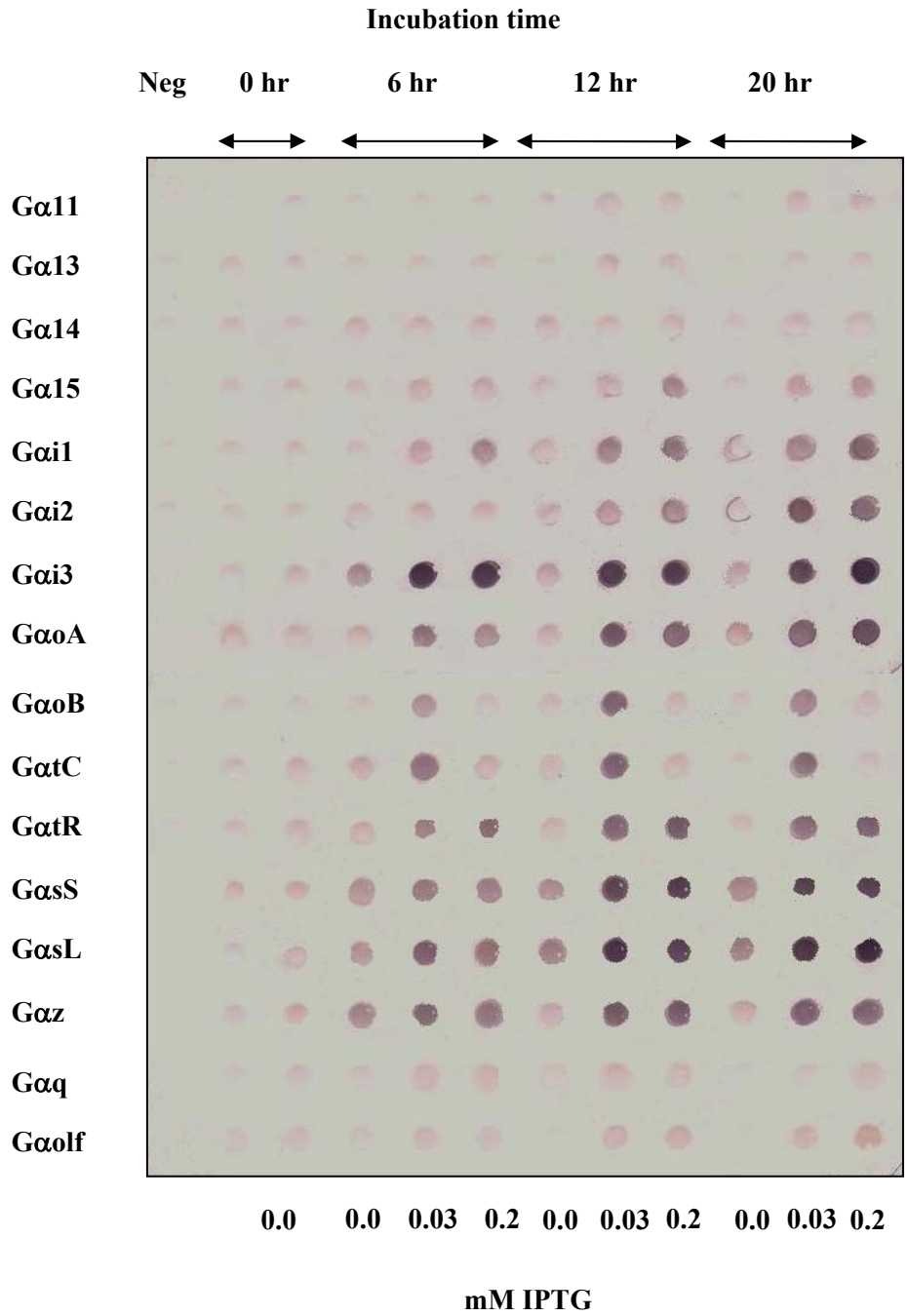


Fig 3.3: Anti-his immuno dotblot screen showing production of Gα proteins in *E. coli.* 16 G proteins were checked for production in BL21-CodonPlus (DE3) strain of *E. coli*. 100 µl of the supernatant after cell lysis was used for each well of the dotblot apparatus (see materials and methods 2.2.1.5). 11 proteins could be expressed in higher yields. Cells were harvested after 6, 12, 20 hr post induction. Two concentrations of IPTG were used, 0.03 mM and 0.2 mM. Neg stands for the *E. coli* strain without any insert.

The production level of Gα proteins, as assessed from the 20 hr dot intensities shows that $G\alpha_{i3} > G\alpha_{sL} > G\alpha_{sS} > G\alpha_{oA} > G\alpha_{i2} > G\alpha_{z} > G\alpha_{tC} > G\alpha_{tR} > G\alpha_{oB} > G\alpha_{i1} > G\alpha_{15}$.

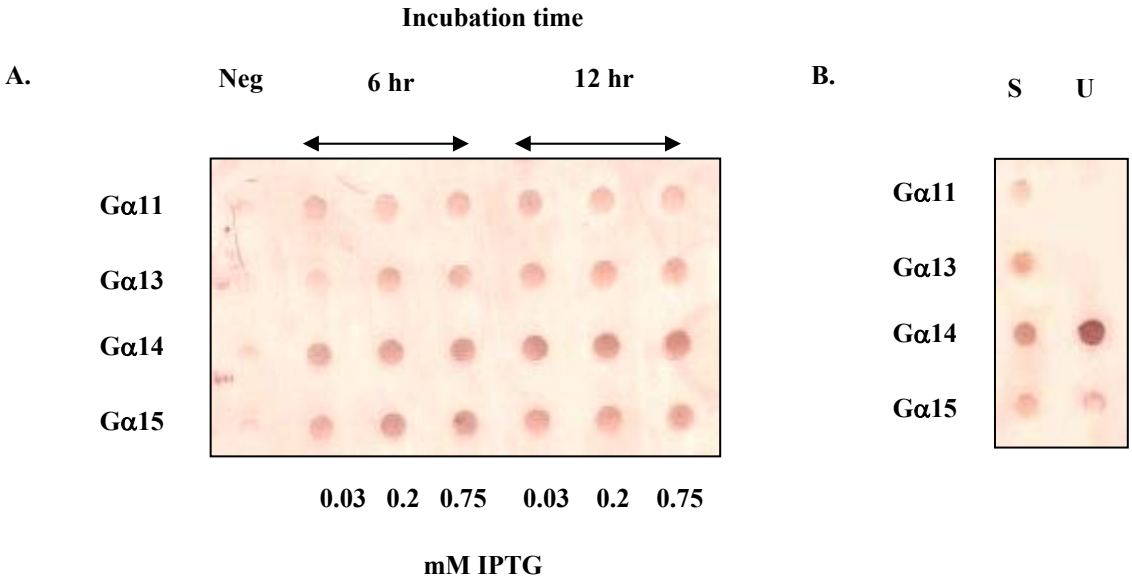


Fig 3.4: Production of Gα subunits in the Rosettagami(DE3)pLysS strain of *E. coli*. A. Those G proteins which could not be produced in the BL21-CodonPlus strain were obtained in Rosettagami(DE3)pLysS strain. $G\alpha_{11}$, $G\alpha_{13}$, $G\alpha_{14}$, $G\alpha_{15}$ showed better production. **B.** $G\alpha_{14}$ was found in insoluble fractions. S represents the soluble fraction of the cell lysate and U represents urea (6M) extract of the pellet fraction. (Anti-his tag antibody immuno dotblot was done similarly as mentioned in Fig 3.3).

3.2.1.1 Production of the $G\alpha_q$ subunit in the yeast *Pichia pastoris.*

The $G\alpha_q$ subunit could not be obtained in *E. coli*. The *P. pastoris* expression system was investigated to express this protein. The $G\alpha_q$ gene (Fig 3.5) was introduced into pPIC3.5K (Appendix A2) vector and transformed into the SMD1163 strain of *P. pastoris*. Protein production was checked by immunoblotting (Fig 3.6). All the colonies selected from the minimal dextrose agar plate contained a band at the expected size (38 KDa), in the immunoblot, indicating the production of G protein.

Fig 3.5: The gene construct used for the Gαq subunit in pPIK3.5 K vector. AOX1 is the alcohol oxidase promoter. His is the hexahistidine tag on the amino terminus.

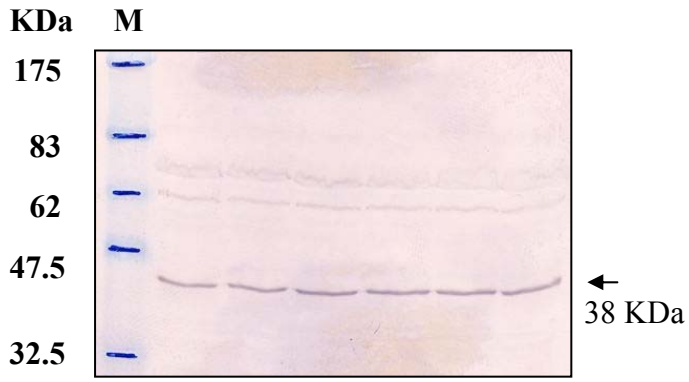


Fig 3.6: Production of the Gα_q protein in *P. pastoris*. M is the marker and six lanes of the gel represent the 6 colonies selected from the minimal dextrose medium. 20 µl of the cell lysate was loaded to each lane. (Anti-his tag antibody immunoblot). Arrow indicates the Gq protein at the expected size.

3.2.2 Production of Gβ and Gγ subunits

5 Gβ proteins were screened for production. Only the $G\beta_1$, $G\beta_{5S}$ and $G\beta_{5L}$ proteins could be obtained in the Rosettagami(DE3)pLysS strain of *E. coli* (Fig: 3.7). The $G\beta_{5S}$ production was less compared to the other two subunits. Protein production was saturated after an incubation time of 8 hr. None of the 11 Gγ subunits screened could be produced in any of the *E. coli* strains used (data not shown).

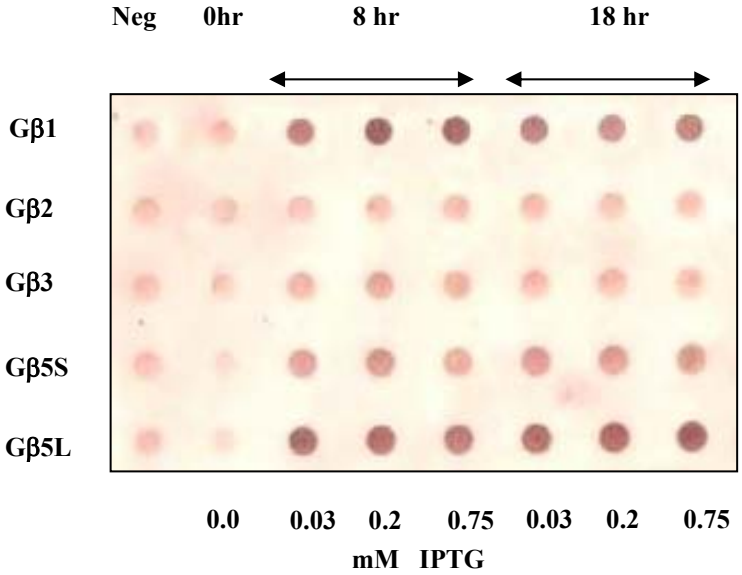


Fig 3.7: Dotblot showing production of Gβ subunits in the Rosettagami(DE3)pLysS strain of *E. coli*. $G\beta_1$, $G\beta_{5S}$ and $G\beta_{5L}$ subunits could be produced in *E. coli*. 8 hr and 18 hr incubations times were tested. 8 hr incubation time was sufficient for protein production. Different IPTG concentrations showed similar production levels. Neg is the Negative control (no gene). (Anti-his tag antibody immuno dotblot).

3.2.3 Co-expression of the βγ subunits in *P. pastoris*

Since the γ subunits could not be expressed separately in *E. coli*, co-expression of the βγ subunits was explored in the yeast *P. pastoris* . The genes for the β and γ subunits were cloned into an *in vitro* multimerisation vector pAO815. The final construct (Fig 3.8) with one gene for β_1 subunit and one gene for γ_5 subunit was transformed into the yeast cells. One copy of each gene was cloned into the vector to maintain the stoichiometry of the subunits.

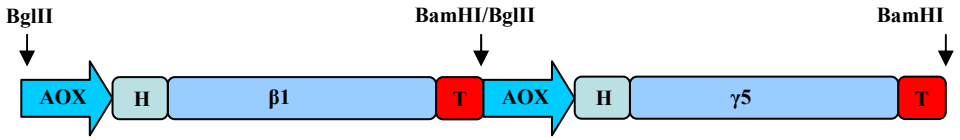


Fig 3.8: The construct for the βγ subunits in pAO815 vector. AOX is the alcohol oxidase promoter, H stands for hexahistidine tag, and T stands for transcription termination sequence. *Bgl*II and *Bam*HI are restriction endonucleases used in cloning.

An anti-his tag immunoblot showed a prominent band, approximately at 42 KDa which is the expected size for the βγ dimer (Fig 3.9). A monomeric form of either subunit could not be seen in the immunoblot.

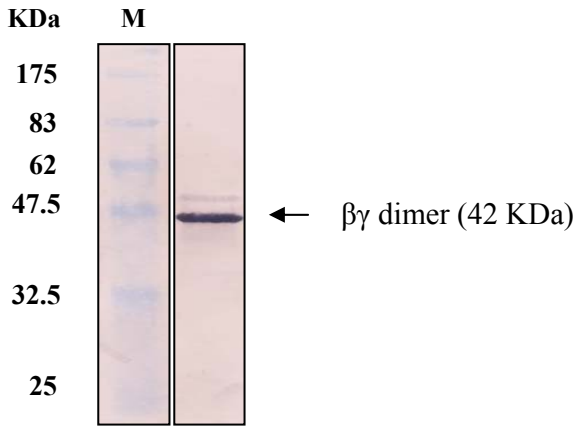


Fig 3.9: Anti-his immunoblot showing production of a βγ dimer in *P. pastoris*. βγ subunits were co-expressed in *P. pastoris*. M denotes the marker. Arrow indicates the βγ dimer band at ~42KDa in an immunoblot (15% SDS-PAGE gel).

3.3 Purification of Gα subunits

$G\alpha_{sL}$ from the G_s family, $G\alpha_{i1}$, $G\alpha_{i3}$, $G\alpha_{oA}$ and $G\alpha_{oB}$ from the $G_{i/o}$ family were produced in the BL21 CodonPlus strain of *E. coli* and purified by IMAC and ion-exchange

chromatography. After Ni-NTA purification the protein was almost 80% pure, as estimated by Coomassie stained SDS-PAGE gel (Fig 3.10). The eluate from Ni-NTA was again purified using the Q-sepharose matrix. All the G proteins investigated eluted at 150-200 mM NaCl. As observed on SDS-PAGE gel of the samples obtained from the Q-sepharose matrix, the protein was more than 90% pure (Fig 3.11). When the protein was used for crystallization, a third step of purification (gel filtration) was used in order to maintain the buffer composition constant from all the batches of purification. The protein eluted at a retention volume of 1.35 ml from a Superose 12 column (Fig 3.12). The $G\alpha_{sL}$, $G\alpha_{i1}$ and $G\alpha_{i3}$ subunits were purified in order to check the coupling with the GPCRs. $G\alpha_{oA}$ and $G\alpha_{oB}$ were expressed in large scale culture and the protein purified was used for crystallization.

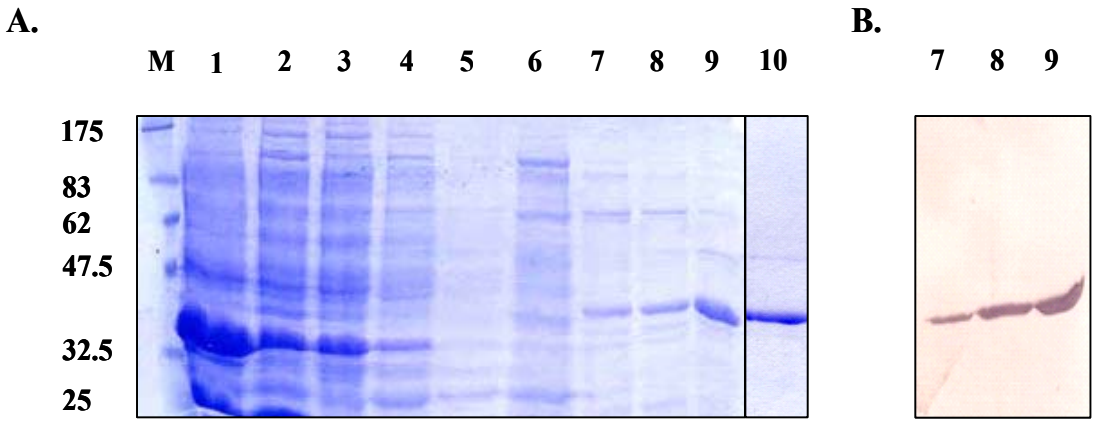


Fig 3.10: SDS-PAGE of purified Gαi1 using a Ni-NTA agarose matrix. A. Coomassie stained 12% SDS-PAGE gel showing: M-marker, 1-cell lysate, 2-flow through, 3-wash with equilibration buffer, 4-wash with 500 mM NaCl buffer, 5-wash with 20 mM imidazole buffer, 6-wash with 40 mM imidazole buffer, 7-9 different fractions of elution with 250 mM imidazole buffer, 10-Protein purified using the Q-Sepharose matrix. **B.** Immunoblot by anti-his tag antibody. 20 µl of different purification fractions were loaded to each well.

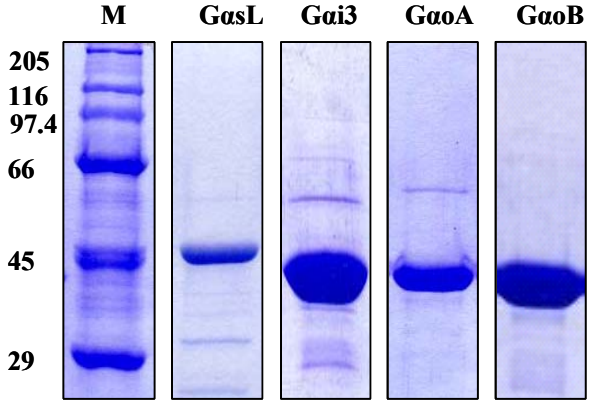


Fig 3.11: G protein samples after a two step purification . Samples of GαsL, Gαi3, GαoA, $G\alpha_{oB}$ were analysed by SDS-PAGE, to assess the purity. A 20 µl sample of the peak fraction from the Q-Sepharose gradient elution was analyzed using a 12% SDS-PAGE gel.

Table 3.1: Yield of pure Gα subunits produced in *E. coli*, by two step purification:

Protein	Yield of pure protein	*E. coli* strain
$G\alpha_{i3}$	10 mg/L	(BL21(DE3) CodonPlus RP)
$G\alpha_{sL}$	8 mg/L	(BL21(DE3) CodonPlus RP)
$G\alpha_{oA}$	5 mg/L	(BL21(DE3) CodonPlus RP)
$G\alpha_{i1}$	3 mg/Liter culture	(Rosettagami(DE3)pLysS)

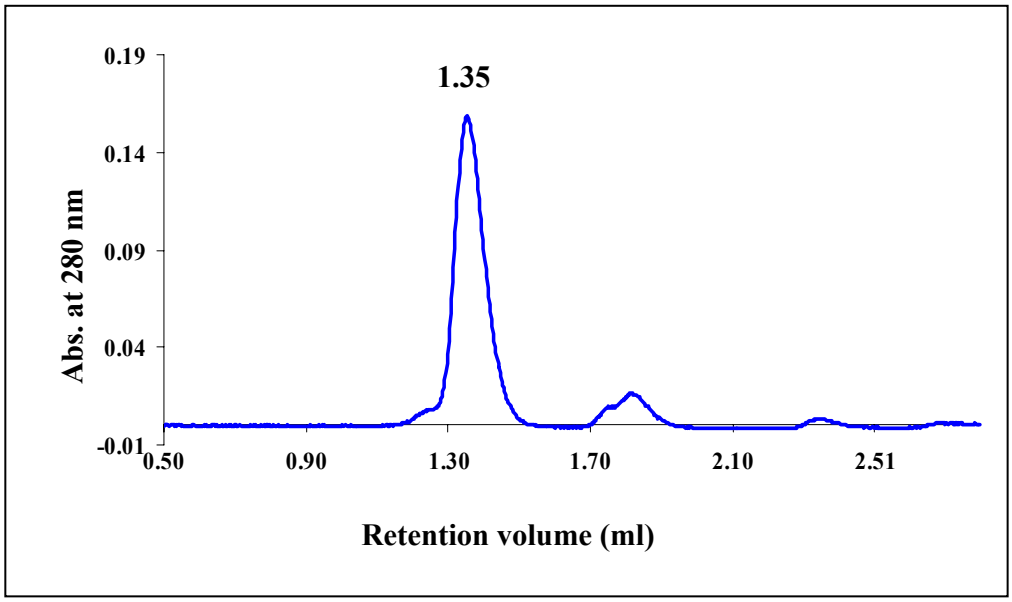


Fig 3.12: Gel filtration profile of the $G\alpha_{oA}$ protein on a Superose 12 column. The protein eluted at a retention volume of 1.35 ml. 100 µl protein sample (~100 µg), purified by Ni-NTA and Q-Sepharose was loaded onto the column. Gel filtration was carried out at 4ºC at a flow rate of 50µl/min.

3.3.1 Purification of G proteins produced in *P. pastoris*

The $G\alpha_q$ subunit was produced in *P. pastoris*. A two step purification of Gq protein yielded approximately 4 mg/L culture. The protein was pure as seen in Fig 3.13.

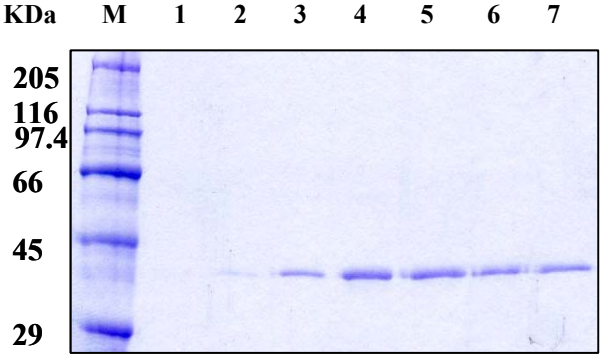


Fig 3.13: SDS-PAGE gel showing Gαq protein, expressed in *P. pastoris*. Each lane of the gel corresponds to one fraction of the NaCl gradient (50-300 mM) applied to the Q-Sepharose column. (Coomassie stained 12% SDS-PAGE gel)

The Gβγ dimer was produced in *P. pastoris*. Most of the protein produced was found in the cytosol rather than in the membrane. The protein was purified from the cell lysate by Ni-NTA chromatography. On SDS-PAGE gels a prominent band was obtained at the size of βγ dimer. There were three major visible impurities two above and one below the dimer band (Fig 3.14). Gel filtration experiments to investigate whether a heterotrimeric complex from G_{oA} and $\beta_1\gamma_5$ dimer formation, did not prove existence of such complex.

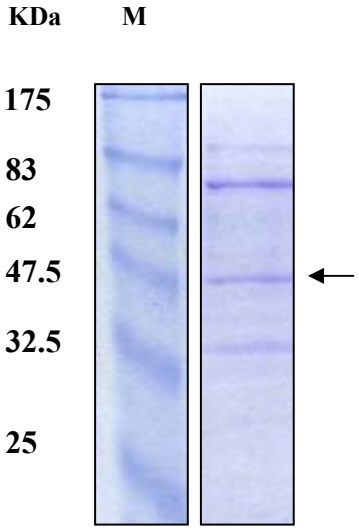


Fig 3.14: SDS-PAGE gel showing the βγ dimer expressed in *P. pastoris*. The arrow shows the dimer complex (42 KDa) of the βγ subunits purified by Ni-NTA, in the Coomassie stained SDS-PAGE gel.

3.4 Fluorescent GTPγS binding assay

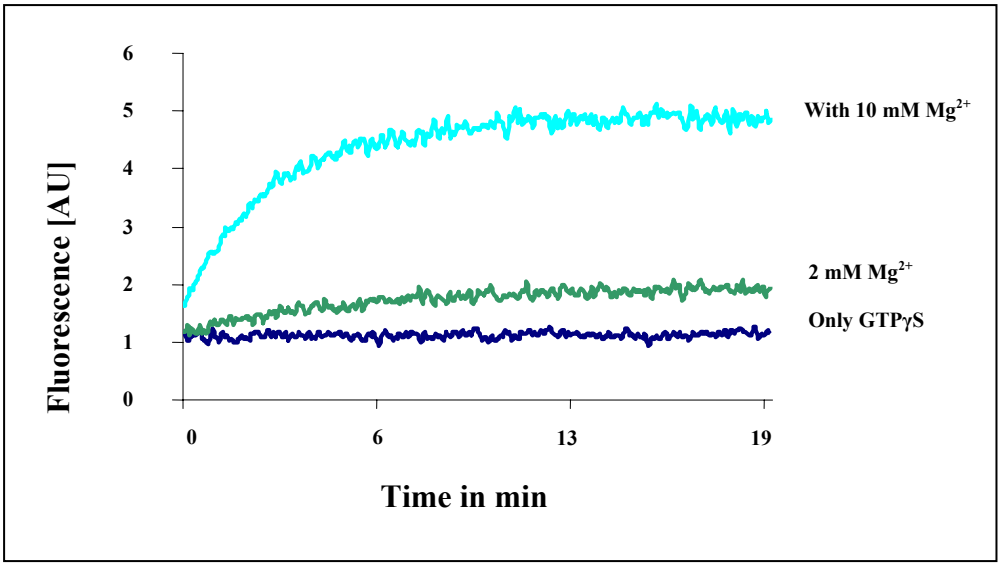


Fig 3.15: Time dependent fluorescence changes showing effect of magnesium on GTP binding. 1µg G protein (G_{sL}) was used to determine the effect of Mg^{2+} on GTPγS binding. Reaction was started with the addition of 1µg pure G protein to the buffer containing 100 nM fluorescent GTPγS. 100 nM fluorescent GTPγS, without protein was used as a negative control.

G protein activity was determined using the fluorescent GTPγS binding assay. Bodipy-FL GTPγS was used as a fluorescent analog of GTP. GTPγS was excited at 485 nm and the emission was detected at 520 nm. Mg^{2+} ion is an important requisite for GTPγS binding to the G protein (Fig 3.15). Different Gα subunits require different concentrations of Mg^{2+} ions. The $G\alpha_s$ protein requires a Mg^{2+} ion concentration of more than 20 mM in the buffer, where as the $G\alpha_o$ protein showed good binding even at 1 mM (data not shown). Increasing concentrations of GDP in the assay reduced the GTPγS binding to the G protein as expected. So GDP was added to reduce the background binding when receptor-G protein interactions were measured.

3.4.1 Effect of detergent on GTPγS binding to G protein

An interesting result of the GTPγS binding assays was the effect of detergents on the GTPγS binding to the G protein. In general, detergents had a negative effect on GTPγS binding. The $G\alpha_s$ protein was more sensitive to detergents among the G proteins tested. $G\alpha_{i/o}$ family members remained active at detergent concentrations which abolished $G\alpha_s$ protein activity (Fig: 3.16). Detergents at concentrations below their CMC had very little effect. But as soon as the concentration of the detergent was increased to its CMC or above, the protein tends to lose its activity. Polar detergents and non-polar detergents both destroyed the activity. Detergents like laurylmaltoside, octylglucoside, octylmaltoside, LDAO, Foscholine12, Tween 20, digitonin were checked, all of them prevented GTPγS binding (not shown).

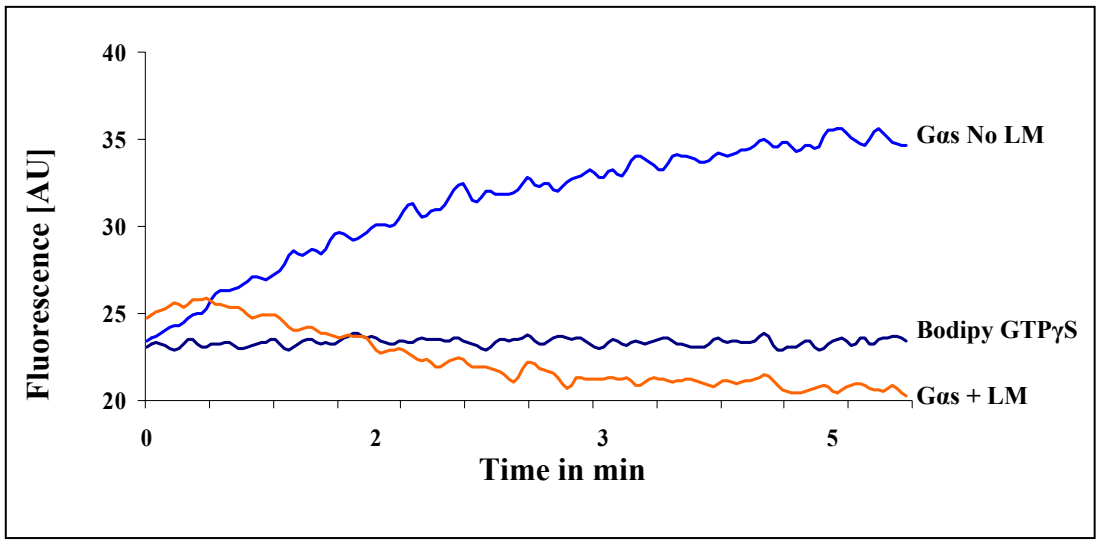


Fig 3.16: Effect of detergent on GTPγS binding to G protein as measured by fluorescence. $G\alpha_s$ (1µg) was added to 500nM Bodipy GTPγS to start the reaction. Presence of 0.1% LM prevented the GTPγS binding.

In order to determine whether the loss of activity was permanent or reversible, the $G\alpha_s$ protein was added to buffer (methods 2.2.6) containing 0.1% LM. The protein solution was incubated for 1 hour and then the protein was purified by gel filtration to remove the detergent from the protein sample (Fig 3.17). The protein from the gel filtration was again checked for the activity. It was found that the removal of the detergent from the solution restored the activity of the G protein.

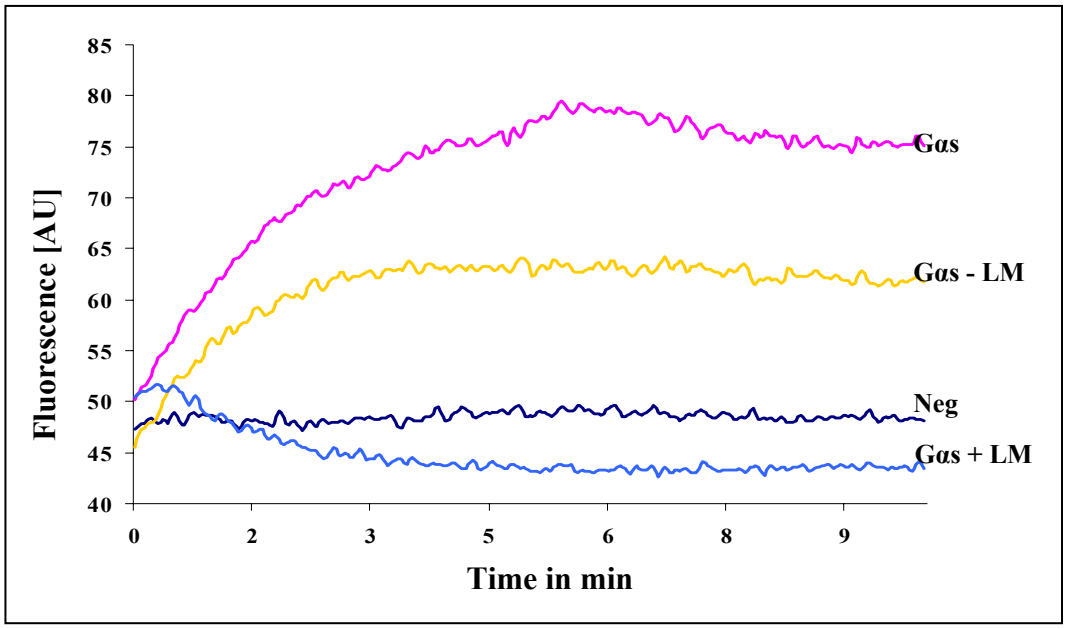


Fig 3.17: Effect of LM on GTP binding activity of GαS protein. Gαs (4 µg) shows the activity with out detergent. Neg denotes the negative control which is the fluorescent GTPγS ligand (500nM) in buffer. $G\alpha_s$+LM shows the loss of G protein activity when 0.1% LM was included in the reaction. $G\alpha_s$–LM represents activity after removing the detergent, by gel filtration. Reactions were started by the addition of protein to buffer with GTPγS.

The intrinsic fluorescence of the protein was measured for Gαs protein to determine the conformational changes occurring upon the addition of detergent. Fluorescence of the protein due to tryptophan was measured for this purpose. The protein was excited at 280 nm and the emission was monitored at 340 nm (Fig: 3.18). If there are any changes in the conformation of the protein there will be a change in the tryptophan fluorescence, because of the exposure of hidden tryptophans. As expected in this case with the addition of detergent (0.1% LM) the protein ($G\alpha_s$) has undergone a conformational change which resulted in an increase of the intrinsic fluorescence. This change was observed in those proteins which lose their activity upon addition of detergent. $G\alpha_s$ protein showed an increase in intrinsic fluorescence where as $G\alpha_i$ protein did not show any change.

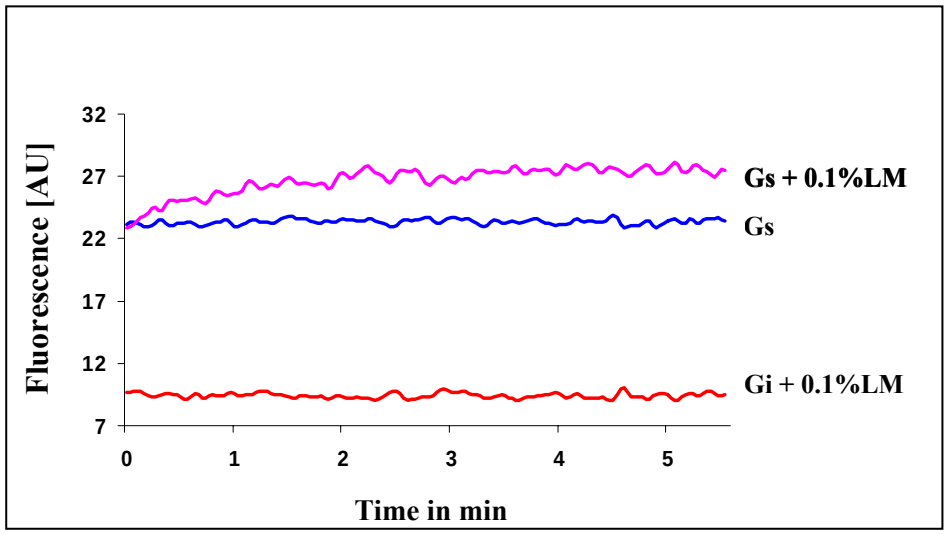


Fig 3.18: Intrinsic fluorescence of G protein. G protein, Gs (10 µg) was mixed with a buffer containing detergent (0.1% LM) to start the reaction and the intrinsic fluorescence of Tryptophan at 340 nm was monitored by exciting the protein sample at 280 nm.

3.5 Crystallization of G proteins

G protein crystallization trials were carried out using the crystal screens from Hampton Research, Jena Biosciences and Nextal. The crystallization technique used was vapour diffusion. Crystallization drops were set up in a 24 well plate or a 96 well plate depending on the number of screening conditions. In a 24 well plate a 3+3 µl drop of protein and buffer was setup as a hanging drop. When using large number of crystal screens, a 0.5+0.5 µl drop was set up as a sitting drop in a 96 well plate. For setting drops in 96 well plate a robotic system (Cartesian tech.) was used. Plates were incubated at 18ºC. $G\alpha_{oA}$, $G\alpha_{oB}$ and $G\alpha_{q}$ proteins were used in crystallization experiments. $G\alpha_{oA}$ and $G\alpha_{oB}$ never gave any promising hits which could be further optimized. But $G\alpha_{q}$ protein which was produced in the *P. pastoris* expression system gave positive hits from Hampton Research (Screen I, buffer 41). Crystals were observed in a 3+3 µl sitting drop, in a buffer containing 100 mM HEPES-Na, pH 7.5, 10% (v/v) isopropanol, 20% (w/v) PEG 4000. Protein solution was used at a concentration of 10 mg/ml in a buffer containing 20 mM Tris/HCl, pH 8.0, 200 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 50 µM GDP.

Crystals were observed after 2 days at 18ºC. Crystals were like needles/tubes. Crystals were not separate but seemed to be stacked on each other (Fig 3.19 A). To check whether the crystals were of protein or salt, they were washed in reservoir buffer and then

analyzed on a SDS-PAGE gel by silver staining (Fig 3.19 B). SDS-PAGE of the crystals gave a protein band at the expected size (38 KDa). Crystals were checked for diffraction synchrotron radiation (ESRF, Grenoble). Crystals diffracted to about 6.5 Å (Fig 3.19 C). The spots obtained were not prominent but diffused or overlapped. The Crystal quality has to be optimized to get better diffraction. At present there is a problem of protein production in *P. pastoris* which has to be sorted out. These experiments became obsolete because the crystal structure had been published by Tesmer *et al*., 2005.

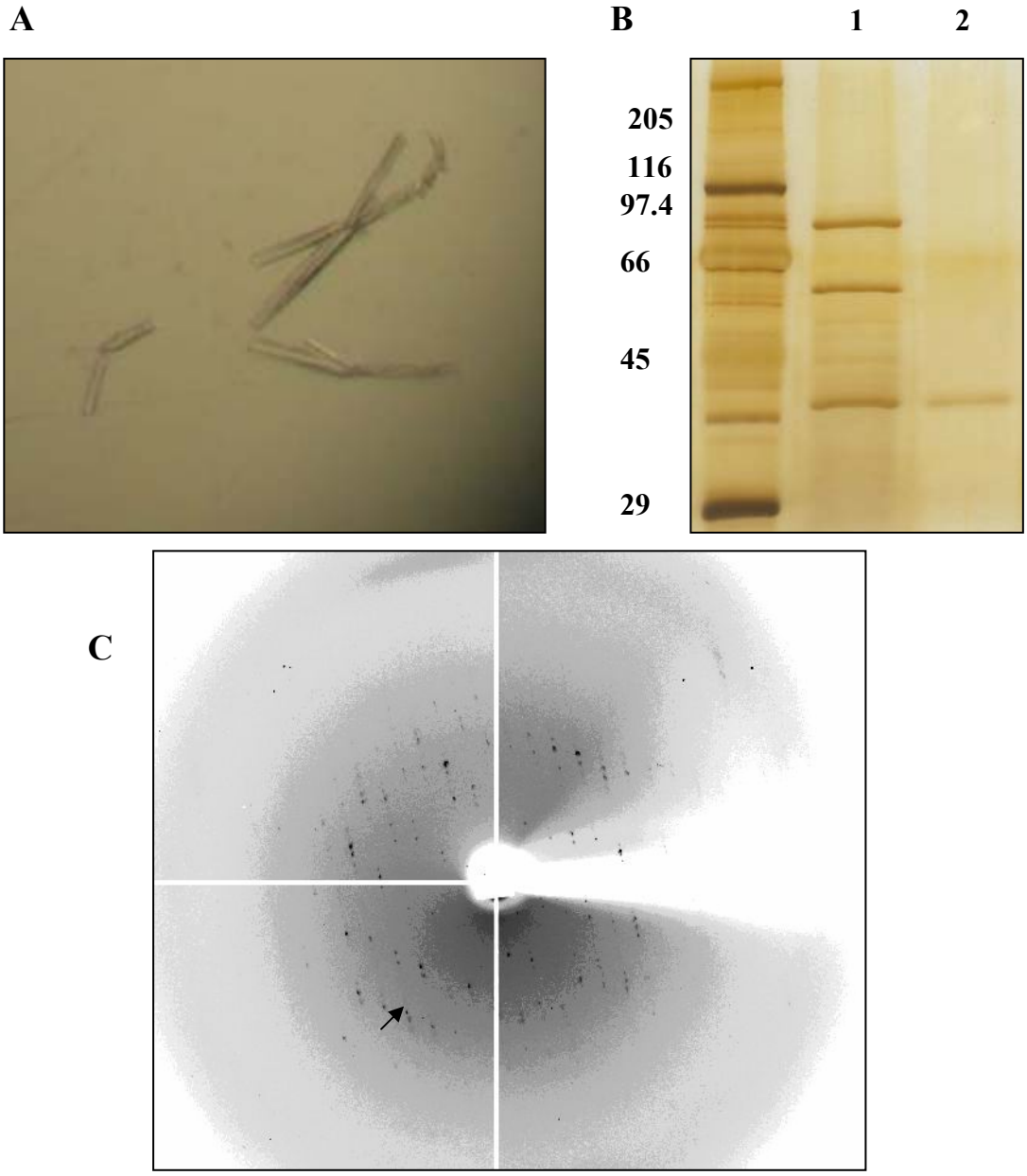


Fig 3.19: Characterization of Gα_q protein crystals. A. 3D Crystals of G protein obtained in a 6 µl drop. **B. Silver stained SDS-PAGE gel.** Lane 1 represents the G protein used for crystallization (left in refrigerator for 3 months after crystallization). The higher bands might contain aggregated protein. Lane 2 contained crystals (100 microns in length) dissolved in loading buffer. **C.** Diffraction pattern at 6.5 Å resolution.

Chapter II: Production and purification of the cannabinoid receptors CB1 and CB2

Production of the cannabinoid receptor 2 (CB2) was first tried in *P. pastoris.* To find a solution for the problems encountered in receptor production in the Pichia system, baculovirus expression system was used later. In the baculovirus expression system both CB1 and CB2 receptors were produced. A detailed characterization was carried out for the CB1 receptor because of its better biochemical characterization and its importance in the pharmaceutical industry.

3.6 Production of the cannabinoid receptor 2 in *P. pastoris.*

The gene for the cannabinoid receptor 2 was cloned in pPIC9K vector and initial expression screening was performed in the MEPNET program. A 96 well plate containing the cultures of 96 colonies from MD plates was obtained from MEPNET team (Andre *et al.*, 2006). A colony which gave a high expression signal on immunoblot was chosen for further investigations. The gene construct in pPIC9K contained an N-terminal Flag epitope, a decahistidine tag followed by the receptor gene and a biotinylation domain of the C-terminus (Fig 3.20). Receptor cloning into the vector was done using *Bam*HI (frame is nnG GAT CCn) next to TEV cleavage site and EcoRI (GAA TTC) restriction sites.

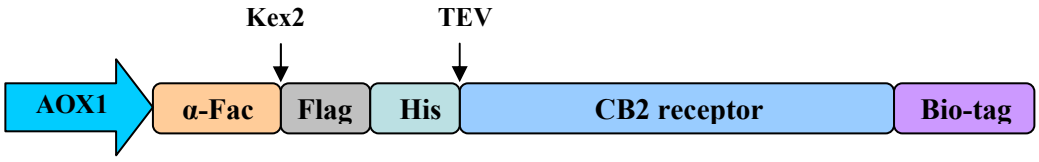


Fig 3.20: Gene construct for CB2 receptor in pPIC9K vector. AOX1 stands for Alcohol oxidase promoter, α-Fac stands for α-Factor secretion signal, Flag stands for Flag epitope, His stands for decahistidine tag, Bio-tag stands for biotinylation domain, Kex2 stands for Kex2 protease signal cleavage site, TEV stands for Tobacco etch virus protease cleavage site.

The cannabinoid receptor 2 was produced in *P. pastoris*, cultured in 5 liter baffled flasks. Membranes were prepared from the cells as discussed in methods section (2.2.2.4). Cell membranes showed specific CB2 ligand binding. Because of the high background

binding of the radiolabeled ligand [H^3] CP-55,940 and non-saturable binding, determination of Bmax and Kd was not very precise.

3.6.1 Solubilization and purification of the CB2 receptor.

To determine the efficiency of solubilization by different detergents, a small scale solubilization screening was performed. 200 µl aliquots containing cell membranes at 5 mg/ml concentration and 1% detergent was incubated under slow rotation, in a cold room for 1 hr. The reactions were ultra-centrifuged at 150,000xg for 30 min. The clear supernatant was analysed by SDS-PAGE followed by immunoblotting.

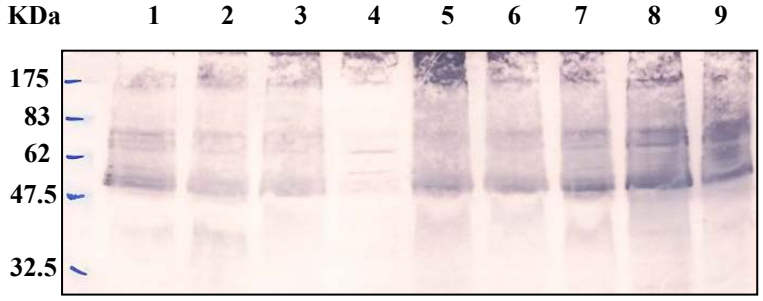


Fig 3.21: Solubilization screening of the cannabinoid receptor 2 (Anti-flag M2 Immunoblot). The gel shows: 1. 1 % laurylmaltoside, 2. 1 % octylmaltoside, 3. 1 % laurylsucrose, 4. 1 % octylglucoside, 5. 1 % dodecylamine-N-oxide, 6. 1 % LM + 0.2% Fos 12, 7. 1 % Foscholine 12, 8. 1 % Foscholine 14, 9. SDS. 20µl of the solubilizate was loaded to each lane of the 12% SDS-PAGE gel.

The CB2 receptor could be solubilized in most of the detergents screened, except octylglucoside (Fig 3.21). An immunoblot of the solubilized samples showed extensive aggregation and many oligomeric states. Laurylmaltoside was used for the purification of receptor. About 100 mg (10ml) of yeast cell membranes ware solubilized with LM at 4ºC for 1 hr. The clear supernatant after ultra-centrifugation was loaded onto Ni-NTA agarose, in a batch process. The purification was performed as discussed in the methods section (2.2.7). Eluate from the Ni-NTA material was not pure (Fig 3.22 A). There were many aggregation bands visible on the immunoblot even after the Ni-NTA purification. Monomeric avidin affinity chromatography was used as a second purification step. In the second stage of purification using the monomeric avidin agarose, most of the protein was found to be bound to the column material and could not be specifically eluted using biotin. The yield of protein eluted from the monomeric avidin matrix was less than 10% of the total receptor calculated (~30 pmol/mg) in the membrane. The protein which could be

eluted from the monomeric avidin column was not pure or homogenous (Fig 3.22 B). Several aggregation bands were still prominently visible in SDS- PAGE gels.

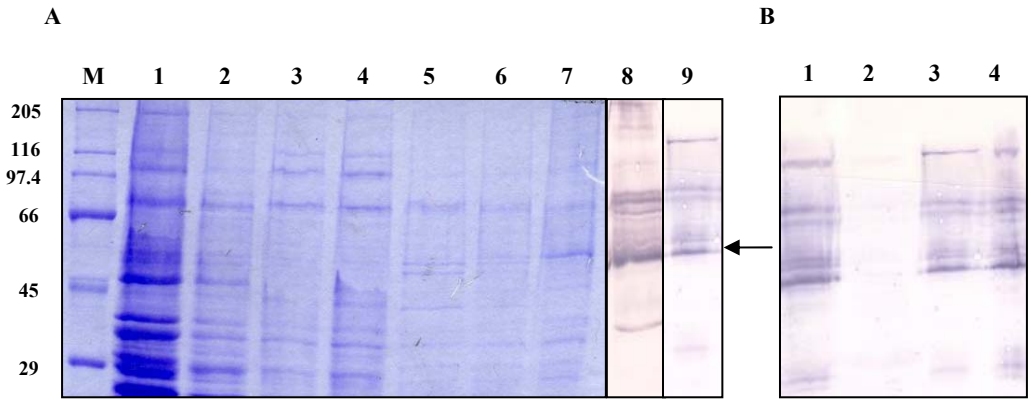


Fig 3.22: SDS-PAGE showing purification of the CB2 receptor from *P. pastoris* membrane. A. Coomassie stained SDS-PAGE gel shows M. High molecular weight marker, 1. cell membrane, 2. solubilizate, 3. wash with equilibration buffer, 4. wash with high salt buffer, 5. wash with 20 mM imidazole, 6. wash with 40 mM imidazole, 7. elution with 350 mM imidazole, 8. Anti-flag M2 immunoblot of Ni-NTA eluate, 9. Strep-AP immunoblot of elute from monomeric avidin. **B.** Streptavidin alkaline phosphatase immunoblot of samples from monomeric avidin purification. 1. Ni-NTA eluate loaded onto monomeric avidin agarose matrix, 2. flow through from the column, 3. eluate from column, 4. protein eluted during regeneration of the matrix with 0.1 M glycine. Arrow indicates the monomeric form of the receptor. 12% gels were used unless otherwise mentioned.

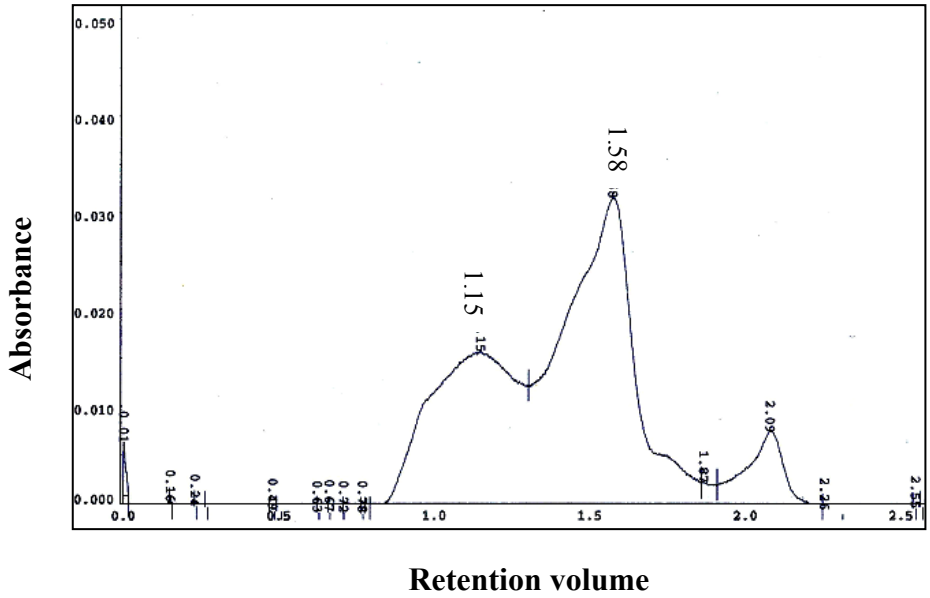


Fig 3.23: Elution profile of the CB2 receptor produced in *P. pastoris*. Gel filtration was done on Superdex 200 column. Receptor was found in the peak at 1.15 ml. 1.58 ml retention volume corresponds to detergent.

The CB2 receptor purified from the monomeric avidin column was analysed by gel filtration to assess the homogeneity of the protein. As evident from SDS-PAGE, the gel filtration profile indicates that the protein was mostly aggregated (Fig 3.23). Several

stabilizing agents like glycerol, sucrose, glycine were added to the buffer starting from the solubilization step but the protein obtained was not homogenous. Several detergents were used to solubilize the protein from the membrane, but didn't show any improvement.

3.7 Expression of cannabinoid receptors in insect cells.

Since the CB2 receptor produced in *P. pastoris* showed extensive aggregation, another expression system was investigated. Baculovirus mediated insect cell expression system was chosen to produce cannabinoid receptors.

3.7.1 Cloning and expression of cannabinoid receptors

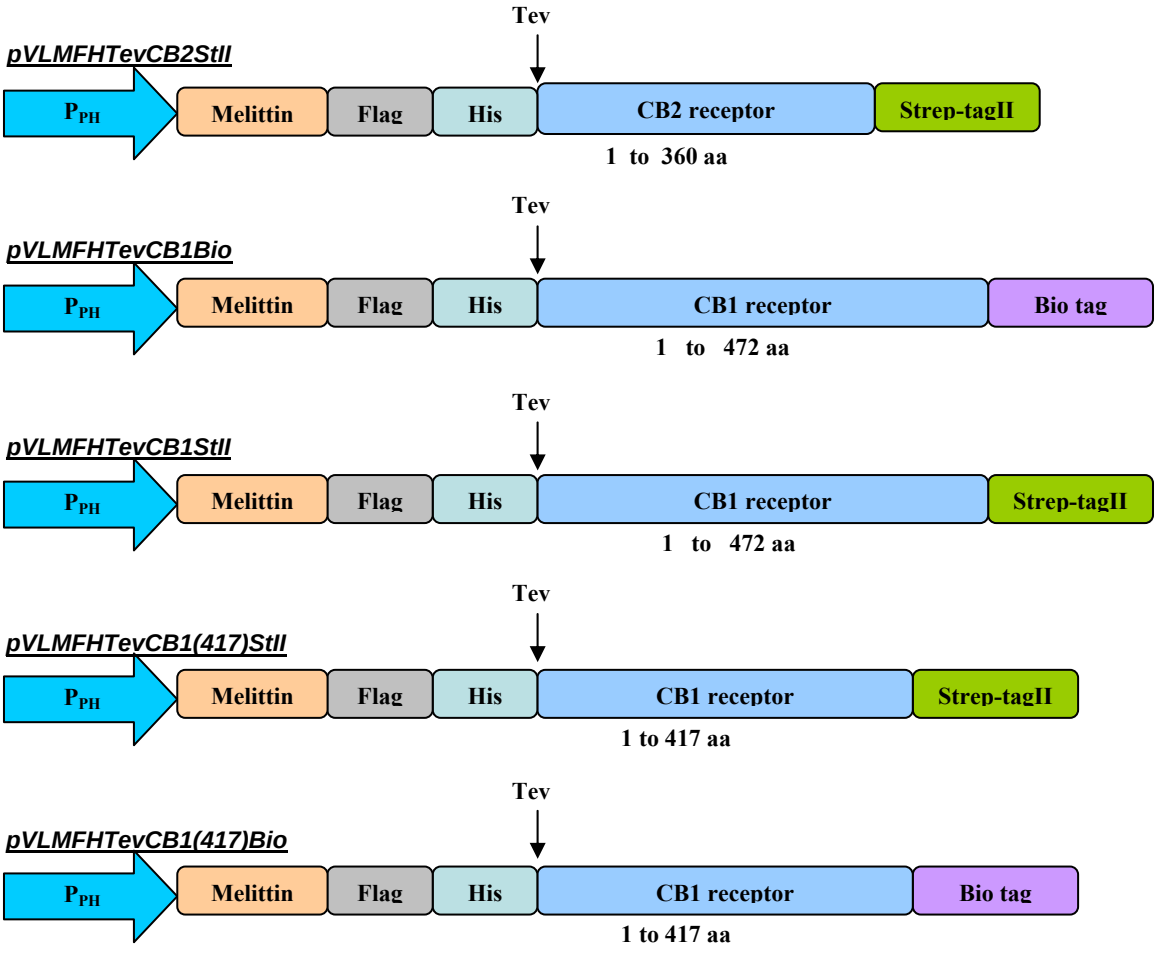


Fig 3.24: Gene constructs used for cannabinoid receptor expression in insect cells. P_{PH} stands for polyhedrin promoter, Melittin (*M*) stands for honey bee melittin signal sequence, Flag (*F*) stands for Flag epitope, His (*H*) stands for decahistidine tag, TEV (*Tev*) stands for tobacco etch virus protease cleavage site, number below CB1/CB2 receptor bar represents the number of amino acids of the receptor cloned and Bio tag stands for biotinylation domain. *StII* stands for strep-tagII.

The CB1 and CB2 receptor genes were cloned into a modified version of the pVL1393 transfer vector, which uses the polyhedron promoter. Different constructs were made to use the receptors for purification and for functional investigations (Fig 3.24). For purification of the receptors decahistidine tags at N-terminus and strep-tagII or a biotinylation domain at the C-terminus were used. A tobacco etch virus protease cleavage site was included after the decahistidine tag, in order to remove the N-terminal tags. Such a cleavage was also used in one of the purification strategies, Ni-Tev-Ni. The DNA sequence of tags and the cloning sites are shown in the appendix (A.9). The molecular weight of the full length CB1 receptor is 52 KDa (without tags) and that of the truncated receptor is 46 KDa. The CB2 receptor has a molecular weight of 40 KDa.

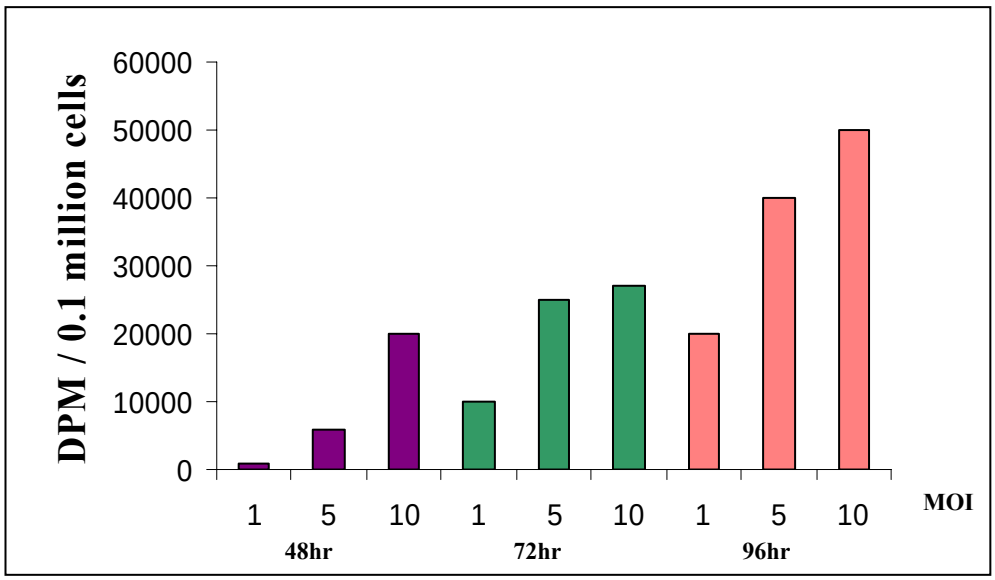


Fig 3.25: Screening of the CB1 receptor (FlagHisTevCB1StrepII) production in Insect cells. 1, 5, 10 MOI virus was tested. Cells were incubated for 48 hr (purple), 72 hr (green) or 96 hr (red) post infection. The Y-axis shows the binding in dpm (disintegrations per minute) of the radioligand (SR141716A) using 100,000 cells per assay.

The amount of virus needed for good expression and the incubation time were also standardized for small scale (10ml) cultures. The CB1 receptor levels ware maximal at 10 MOI, after an incubation time of 96 hr (Fig 3.25). But more cells were lysed after 96 hr, which lead to proteolysis of recombinant protein. So, incubation for 72 hr was generally used to get better protein levels. Different cell lines were verified for expression. *Sf21* and *H5* cells didn't give any better yields (as detected by radioligand binding assay on the same number of cells of each cell-line) compared to *Sf9* cells. All constructs of the receptors could be expressed in the insect cells (Fig 3.26 A). Membranes were first analysed by SDS-PAGE to detect the recombinant protein produced. A radiolabeled ligand binding assay was

used to estimate the amount of functional receptor present in the membranes. Addition of tunicamycin (10 µg/ml culture) to the cell culture produced receptor with of smaller molecular mass (Fig 3.26 B). This shift indicates that the receptor was glycosylated in insect cells.

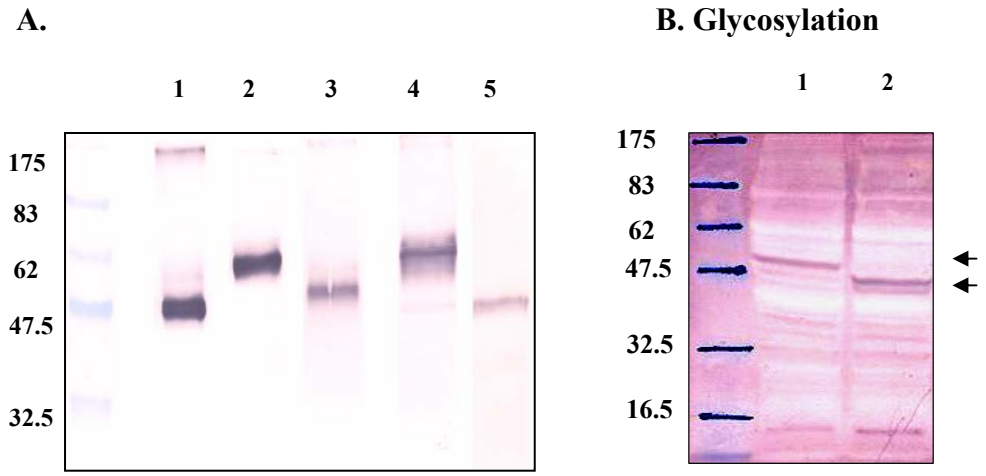


Fig 3.26: Immunoblots showing receptors produced in insect cells. **A.** Anti-flag immunoblot of the membrane (25µg) of *Sf9* cells. 1. C-terminal truncated CB1 receptor (CB1-417) with N-terminal Flag epitope, His tag, TEV cleavage site and C-terminal Strep tag II (FHTCB1-417 St II), 2. FHTCB1-417 Bio tag, 3. FHTCB1 St II, 4. FHTCB1 Bio tag, 5. FHTCB2 St II. **B.** Glycosylation of the receptor. 1. Normal receptor. 2. Receptor from tunicamycin (10µg/ml culture) treated cells showing deglycosylation. The two arrows indicate the shift in receptor band because of deglycosylation. A band around 175 Kda (A. Lane 1) is a cannabinoid receptor specific oligomeric band.

3.7.2 Radioligand binding assay of the CB1 receptor

Functionality of the expressed protein was assessed by the ligand binding assay. The ligands used were either radiolabeled agonists or antagonists. Ligand binding on the membranes was performed in a glass fiber filter assay. Cell membranes were incubated with the radiolabeled ligand in binding assay buffer (20 mM Tris/HCl pH 8.0, 1 mM EDTA, 1% BSA). Since cannabinoids are very hydrophobic and bind non-specifically to the plastic or glass, bovine serum albumin was an important component of the ligand binding reaction, to reduce the background binding of the ligand. Washing of the glass fibre filters was done with the same binding assay buffer warmed to 30˚C. Saturation binding assay was performed to determine the amount of receptor expressed and to estimate the binding constant (Kd) of the receptor. 8 different concentrations of the radioactive ligand were used for the saturation binding assay. Assay reactions contained 1 µg of membrane suspension in the binding assay buffer. 200 µl reactions were setup for each condition. Triplets of positive reactions and duplets of negative reactions with cold ligand were

measured. The mean of the dpm values measured for the samples was used to calculate the amount of receptor present in the membrane in pmol/mg. The data was fitted to a non-linear regression curve, using Kaleida graph software. Specific binding Y=(Bmax * X)/ (Kd+X), where X is the concentration of radioactive ligand, Bmax is the maximum specific binding observed on the membrane, Kd is the dissociation constant of the ligand.

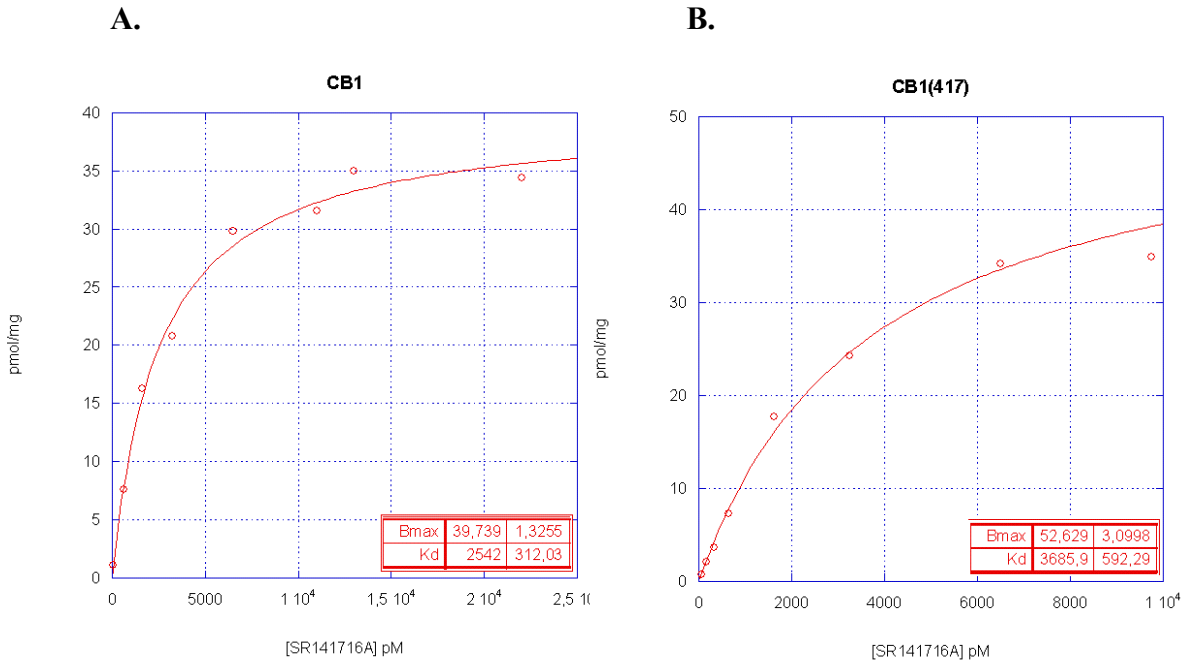


Fig 3.27: Saturation binding curves of CB1 receptor. 1 µg of membrane with FHTevCB1StrepII (**A**) and FHTevCB1(417)StrepII (**B**) receptor was used. The X-axis represents the pM concentrations of radioactive antagonist SR141716A and Y-axis represents receptor concentration in terms of pmol/mg total protein. Bmax value of 40 pmol/mg and a Kd of 2.5 nM were obtained for the full length CB1 receptor. For the truncated receptor FHTevCB1(417)StrepII, Bmax was 52pmol/mg and Kd was 3.6 nM.

Fig 3.27 shows the Bmax and Kd values of FHTevCB1StrepII receptor and the truncated FHTevCB1(417)StrepII receptor. The levels of the truncated receptor ware found to be ~20% higher than that of the full length receptor. The Kd values of both receptor constructs were in the range given in the literature from 0.5 to 7.0 nM for different tissues. The Kd for the full length receptor was 2.5 ± 0.3 nM and for the truncated receptor it was 3.6 ± 0.6 nM. Bmax values of FHCB1StrepII receptor (produced in a bio-reactor at Aventis by Ingo Focken) for agonist was 5 times less than antagonist binding. Table 3.2 shows the Bmax and Kd values of the receptor constructs produced in insect cells.

Table 3.2: Bmax and Kd values for different constructs of the CB1 receptor

Construct	Agonist (H^3 CP-55,940) Bmax (pmol/mg)	Agonist Kd (nM)	Antagonist (H^3 SR141716A) Bmax (pmol/mg)	Antagonist Kd (nM)
FHCB1StII	7.6 ± 0.95	2.4 ± 0.89	38 ± 1.14	2.0 ± 0.14
FHTevCB1StII	ND	ND	39.7 ± 1.3	2.5 ± 0.3
FHTevCB1Bio	ND	ND	20 ± 1.8	4.0 ± 0.8
FHTevCB1 (417)StII	ND	ND	52 ± 3.09	3.6 ± 0.6
FHTevCB1 (417)Bio	ND	ND	17 ± 1.68	2.2 ± 0.58

Ligand binding to the receptor was dependent on the NaCl concentration in the binding assay reaction. The agonist binding was reduced with increasing NaCl concentration, leaving the antagonist binding unaffected (Fig: 3.28). The saturation binding on membranes containing FHTevCB1StrepII receptor, with the agonist (H^3 CP-55940) in the presence of 1M NaCl showed a decrease in the Bmax and a slight increase in the Kd as shown in Table 3.3. Mg^{2+} was absolutely necessary for the agonist binding to the CB1 receptor (Fig 3.28). The antagonist binding was not very much dependent on Mg^{2+} concentration, but increased with high concentrations. DTT had a less detrimental effect on cannabinoid receptors, unlike its effect on many other GPCRs. 30% of the agonist binding was still observed at a DTT concentration of 10 mM (Fig: 3.29) whereas the antagonist binding was decreased only by 10% at 10 mM DTT.

NaCl **Mg^{2+}**

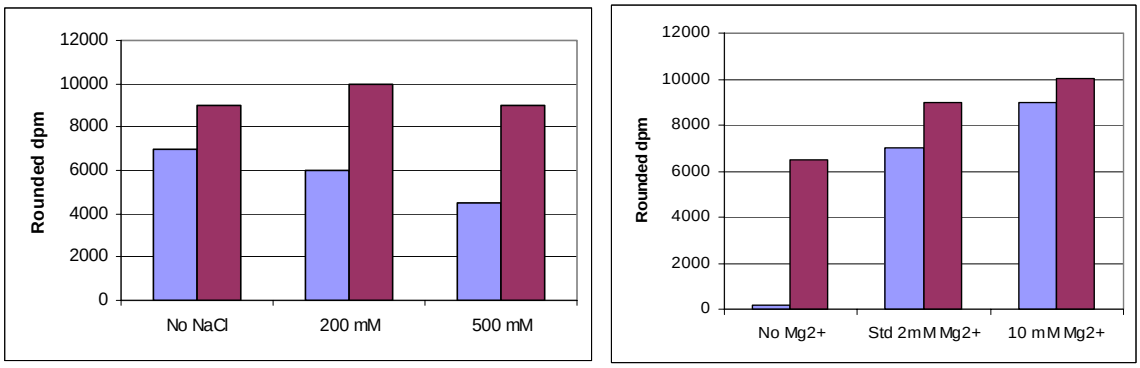


Fig 3.28: Effect of NaCl and Mg^{2+} on ligand binding to the CB1 receptor. Blue bar represents the agonist binding and maroon colour represents the antagonist binding. Rounded dpm refers to the mean dpm values of triplets. 5 µg membrane protein was used in the measurements. 5 nM radioactive ligand was used.

Table 3.3: Effect of NaCl on ligand binding to FHCB1StrepII protein:

FHTevCB1StrepII	100mM NaCl	1M NaCl
Bmax (pmol/mg)	7.6 ±0.95	5.2 ±0.51
Kd (nM)	2.4 ±0.89	3.7 ±0.9

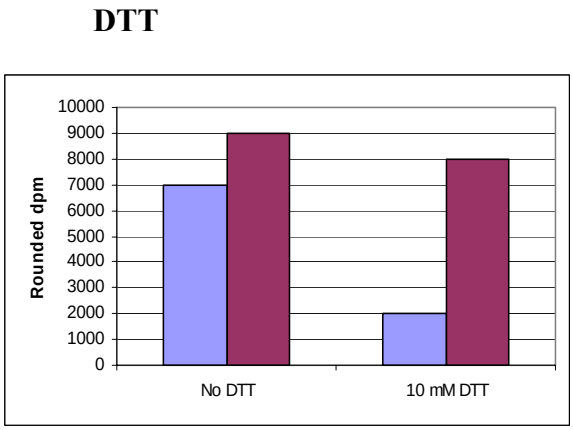


Fig 3.29: Effect of DTT on ligand binding to the CB1 receptor. Cell membranes were incubated with the ligand in a buffer with 10 mM DTT, to observe the effect on ligand binding. 5 µg membrane was used in the measurements. 5 nM radioactive ligand was used.

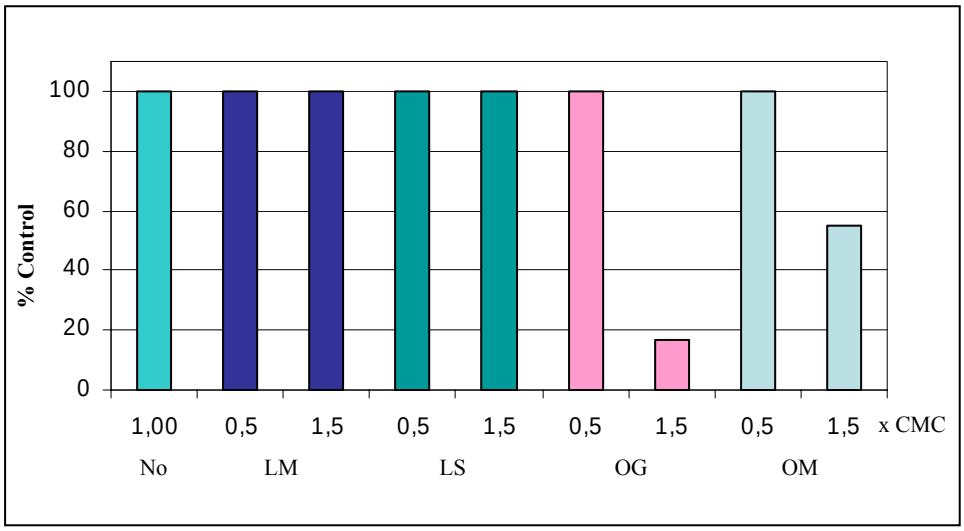


Fig 3.30: Effect of detergent on ligand binding to the CB1 receptor. "No" means no detergent included. LM stands for laurylmaltoside, LS stands for laurylsucrose, OG stands for octylglucoside, OM stands for octylmaltoside. 0.5 and 1.5 represents the number of times the CMC of the detergent. No specific activity was observed when detergents like LDAO, Fos12, Fos14 were used.

Different detergents had varying effects on the binding of the ligand to the CB1 receptor (Fig 3.30). Cell membranes were filtered on glass fiber filters and detergent solutions in the binding assay buffer at a concentration of 0.5 or 1.5 times CMC was added onto it as a first wash. Filters were washed with buffer without detergent and bound ligand was estimated.

	Ligand in	Binding by Hot Agonist
CB1	Agonist	+
	Antagonist	-
CB1(417)	Agonist	+
	Antagonist	-

Fig 3.31: Effect of the presence of ligands during cell culture on the binding assay. CB1 and CB2 receptor agonist WIN55,212-2 or antagonists AM251 (CB1) and AM630 (CB2) were added to the culture during protein production. The binding was checked with radioactive agonist CP55,940. + denotes that the hot agonist binding was possible and – denotes that hot agonist binding was not seen.

When the receptors were produced in the presence of ligands in the culture (Fig 3.31), ligand binding was affected. CB1 receptor agonist WIN 55,212-2 or antagonist AM251 (1 µM concentration) were added to the culture, 24 hr post infection. After the incubation time, the ligand binding was checked on the cells and also on the membranes. When agonist was present in the culture, binding of radioactive agonist (CP 55,940) was unaffected. But when antagonist was present in the culture, radioactive agonist binding was dramatically reduced or absent. This effect was seen both on cells and membranes, which were prepared after several washes with buffer.

3.7.3 Purification of the cannabinoid receptor 1

Purification of cannabinoid receptors has been a challenge for years. Several research groups tried to purify the receptor expressed in different expression systems ranging from *E. coli* to *P. pastoris* to insect cells. But the attempts were not successful to obtain yields sufficient for protein structure determination using 2D or 3D crystallography. Further more, harsh conditions like washing with 8 M Urea were used in some of the

purification procedures. Using such conditions could destroy the activity of the protein. So in this work several new purification strategies were explored.

3.7.3.1 Ni-Tev-Ni purification

In this purification procedure, the receptors were purified using Ni-NTA agarose. Then the N-terminal decahistidine tag was cleaved off using TEV protease. The proteolysed protein was again loaded onto the Ni-NTA agarose matrix. Since the receptor now lacks the His-tag, it elutes in the flow through. TEV protease binds to the matrix, since it possesses a His-tag. Non-specifically bound proteins would still bind to the matrix.

The CB1 receptor could be solubilized in almost all detergents tested (LM, OM, DM, OG, LS, LDAO, Fos12, Fos14, Fos16, $C_{12}E_8$, cymal6). Since the receptor didn't lose its binding in the presence of 1.5 times CMC of LM as discussed before, it was chosen for further solubilization and purification experiments. Insect cell membranes ware solubilized in the presence of 1% LM at 4ºC for 1 hr. The flow through from the Ni-NTA material, in the second round of loading was collected and analysed for purity (Fig 3.32). The protein obtained after Ni-Tev-Ni purification was impure with many non-specific bands seen in SDS-PAGE gel. All the purified proteins showed similar purification profiles.

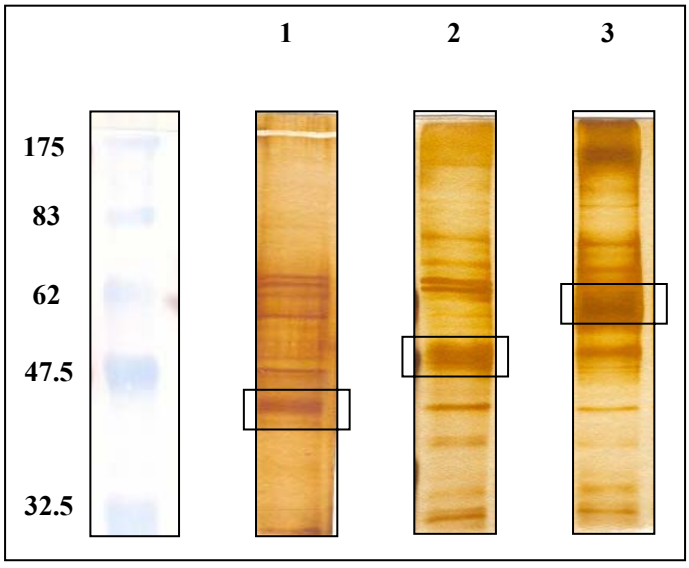


Fig 3.32: Analysis of the purification of cannabinoid receptor by the Ni-Tev-Ni strategy. 1. Truncated CB1 receptor (CB1-417 Strep-tagII) after Ni-Tev-Ni purification analysed on SDS-PAGE (12%) and stained by silver. 2. Full length CB1 receptor (CB1 Strep-tagII), 3.CB1 Bio-tag. Approximately 1 µg of protein was loaded to each well.

3.7.3.2 Ni-NTA and Streptactin purification

In this purification method, protein was first purified by IMAC using Ni-NTA. Eluate from IMAC column was loaded onto the Streptactin agarose, washed and the protein was eluted with 10 mM desthiobiotin. Different fractions of the eluate were analysed by SDS-PAGE, to monitor the purity of protein (Fig 3.33). Most of the protein was found to be not binding to the Streptactin matrix. Most of the protein was found in the flow through. So the yield of the protein after Streptactin chromatography was very poor. But the purity was much better compared to Ni-Tev-Ni purification. Two major impurities were observed above the receptor band, in SDS-PAGE gel.

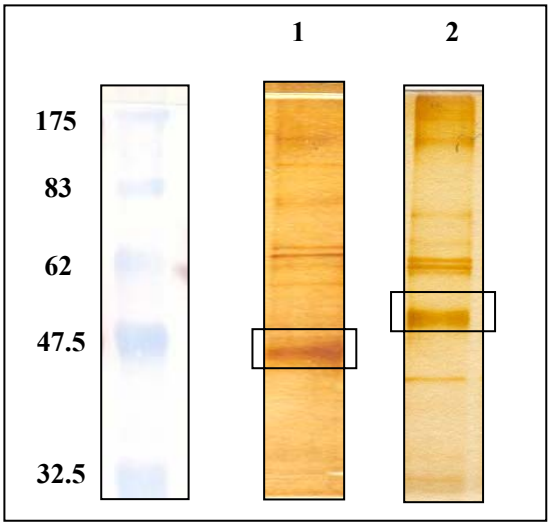


Fig 3.33: SDS-PAGE showing purification of the CB1 receptor using His-tag and Strep-tagII. 1. The truncated receptor (CB1-417 Strep-tagII) after Ni-NTA and Streptactin purification analysed on SDS-PAGE and silver stained. 2. Full length receptor (CB1 Strep-tagII).

3.7.3.3 Ni-NTA and monomeric avidin purification

Receptor with the biotinylation domain (Bio-tag) was purified using IMAC as a first purification step followed by immobilized monomeric avidin affinity chromatography. The eluate from Ni-NTA column was incubated with monomeric avidin material in a cold room for 1 hr. The material was packed into the column and washed with 10 column volumes of buffer. The bound protein was eluted using 10 mM D-biotin. The eluted fractions were analysed on SDS-PAGE to assess homogeneity (Fig 3.34). Most of the protein was found to be irreversibly bound to the monomeric avidin matrix thus decreasing the yield of the purification. This protein could only be removed from the material with glycine/HCl buffer pH 2.8.

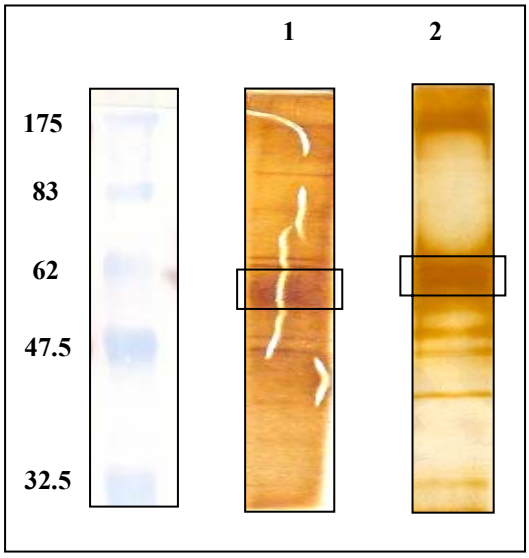


Fig 3.34: Purification of cannabinoid receptor using His-tag and Bio-tag. 1. Truncated CB1 receptor (CB1-417 Bio-tag), 2. The full length CB1 receptor (CB1 Bio-tag) analysed by silver stained SDS-PAGE.

Purified receptor was analysed by gel filtration to assess the homogeneity of the protein. Protein purified by any of the above discussed methods didn't yield absolutely pure protein. So, as expected the gel filtration profile of the receptor didn't show a symmetric peak. A representative gel filtration profile of the CB1 receptor purified by Ni-NTA followed by Streptactin is shown below (Fig 3.35). The broad distribution of the peak might be a result of the other high molecular weight impurities.

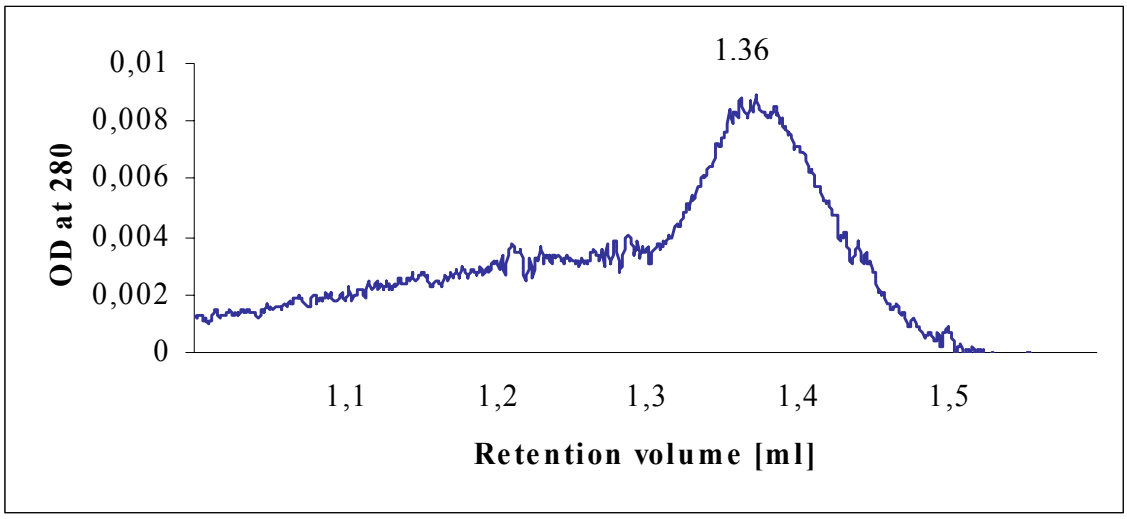


Fig 3.35: Gel filtration profile of purified CB1-StrepII protein. The CB1 receptor purified by Ni-NTA and Streptactin affinity chromatography was analysed using Superose 12 column. A prominent peak was obtained at 1.36 ml which corresponds to the receptor as confirmed by Western blotting. Approximately 10 µg of protein was loaded onto the column.

3.7.3.4 Purification of the CB1 receptor using different IMAC resins

Since the protein yield after the second column was very small, metal affinity chromatography was optimized to get better purity. Different IMAC resins were tried to achieve better purity and yield. Ni-NTA agarose from Qiagen, His-Select from Sigma, Profinity-IMAC from Bio-Rad were tested to find out the best matrix for the receptor purification. Decylmaltoside was used for these studies instead of laurylmaltoside (DM was used by Yeliseev *et al*., 2005, for the CB2 receptor purification). The protein purified using Ni-NTA agarose was impure compared to the protein purified using His-Select or Profinity-IMAC (Fig 3.36). Most of the protein was lost in the flow through from Profinity-IMAC resins. His-Select contains lower density of Ni ions on the matrix. Protein was lost in the flow through, as in the case of Profinity-IMAC, but the eluate contained less impurities compared to the other two resins. So, His-Select was chosen to further optimize the purification.

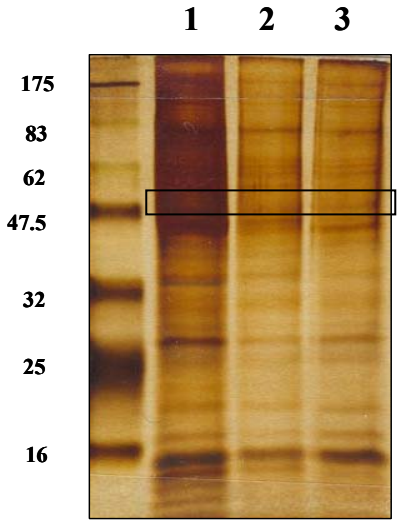


Fig 3.36: comparison of purification of the CB1 Strep-tagII receptor on different IMAC resins as analysed by SDS-PAGE. 1. The CB1 receptor purified from Ni-NTA agarose resin from Qiagen, 2. His-Select from Sigma, 3. Profinity-IMAC from Biorad. A 12% SDS-PAGE gel was used for analysis and it was silver stained.

Protein purification was performed on His-Select material, to further optimize the washing conditions, to improve the purity of the protein. Solubilizate containing the receptor was incubated with His-Select material in a cold room for 1 hr. The material was packed into a column and washed with 10 column volumes of buffer containing 500 mM NaCl, followed by 10 column volumes of buffer containing 20% glycerol. The column was washed with 50 column volumes of buffer containing 30 mM imidazole, to get considerably pure protein. The protein was eluted with 200 mM imidazole. The eluate was analysed using SDS-PAGE. Protein was identified using the epitopes on the protein (Fig 3.37- B). Anti-his tag antibody, Anti-flag tag M2 antibody and Streptavidin-alkaline

phosphatase were used to detect the full length protein. The Streptavidin-AP blot showed a lower band which might be the truncated receptor. Fig 3.37-C shows the N-terminal truncated receptor in the cell membrane as well. Almost 50% of the receptor is therefore without N-terminus and so most of the protein is lost without binding to IMAC matrix. The protein yield was less, with this procedure of purification as well because most of the protein was lost in the flow through and in the excess washing steps (Fig 3.37-A). Never the less the protein obtained was much pure compared to the other purification strategies.

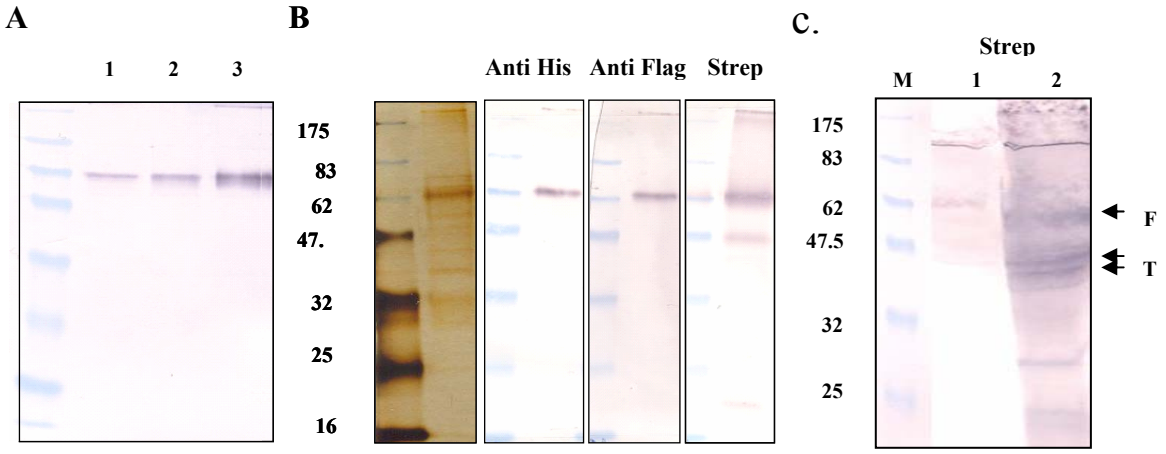


Fig 3.37: Analysis of the purification of the CB1-417 Bio-tag receptor using His-Select IMAC resin on SDS-PAGE and immunoblots. **A.** 1. Flow through from His-Select column, 2. Wash with 30 mM imidazole, 3. Elution with 200 mM imidazole. **B.** Silver stained SDS-PAGE was shown followed by anti-his tag antibody immunoblot, anti-flag M2 antibody blot, and streptavidin alkaline phosphatase blot. The lower band (45KDa) in the streptavidin blot might be the N-terminal cleavage product of the receptor. The high molecular weight band (175 KDa) is an oligomeric form of the receptor. **C**. Streptavidin-AP blot of CB1(417)Bio tag receptor in membrane. F stands for the full length protein, T stands for truncated protein. 1. Sf9 membrane 2. FHTev CB1(417) Bio membrane.

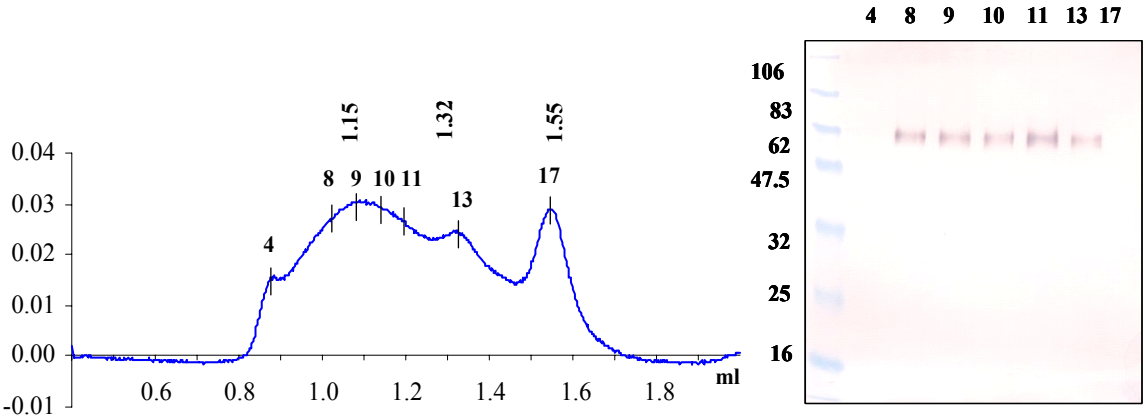


Fig 3.38: Gel filtration of the CB1-417 Bio protein purified from His-Select. Protein was analysed on Superdex 200 column. 30 µg of the purified protein was used. Numbers adjacent to the lines on the chromatogram indicate the fractions analysed by immunoblot of SDS-PAGE using the anti-flag M2 antibody.

The protein was analyzed by gel filtration on a Superdex 200 column (Fig 3.38). There was no clear peak. A major peak was seen at a retention volume of 1.15 ml and a minor peak at 1.32 ml. Both peaks contained the protein. There was no protein detected in the void volume, which means the protein was not aggregated. The broad peak might be a result of impure protein and possible oligomeric forms of the protein.

Table 3.4: Purification yield of the CB1(417) receptor

Purification	*Protein recovered*	*% of total*
Membrane	150 µg receptor	100%
Ni-NTA	NA(Impure)	NA
His-Select *(417-Bio)*	30 µg protein*	20%
Streptactin	25 µg protein	16%
Monomeric avidin	30 µg protein	20%

{ A receptor of 50 KDa expressed at a level of 30 pmol/mg of total protein is equal to 1.5 µg receptor per 1 mg cell membrane} * Calculated yield 60 µg pure protein/L culture.

The purification yields given above in the table are values obtained from BCA protein quantification. 150µg receptor was calculated based on the Bmax values of the receptor constructs. The protein is not pure enough to determine the exact amount of the receptor present in the sample. Radiolabeled binding assay on solubilized or purified protein is not successful because of the high non-specific binding of the cannabinoid ligands.

3.8 Stable expression of the CB1 receptor in insect cells

Stable expression of protein in insect cells was explored using the "Invitrogen's kit for selection of stably expressing lepidopteran cells". This system uses the early phase promoters from *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus in vector pMIB/V5-His (Appendix A5). The gene coding for the recombinant protein is under control of the *OpIE2* promoter. Membrane protein expression is enhanced by the honey bee melittin secretion signal. Blasticidin S antibiotic is a selection marker which is under

control of the *OpIE1* promoter. After transformation the cells were selected by the antibiotic resistance. But more than 80% of the cells sustained the antibiotic stress, whereas production of recombinant receptor was not confirmed in radioligand binding experiment. Later on the protein was fused at the genetic level to YFP for use as a selection marker. Highly expressing cells were selected using FACS machine. Unexpectedly there were two populations of *Sf9* cells and the cells couldn't sustain the harshness of the cell sorter. The cells didn't grow after sorting. The cells were selected by the dilution method making use of the fluorescent marker itself (Fig 3.39). The presence of the receptor was confirmed by immunoblotting and immunogold labeling experiments (Fig 3.40). It was found that the number of receptor sites on the cells were only 10% of that obtained using the baculovirus expression system.

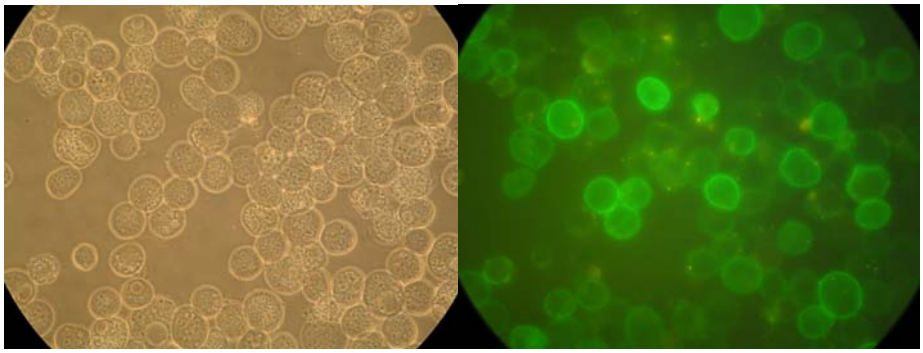
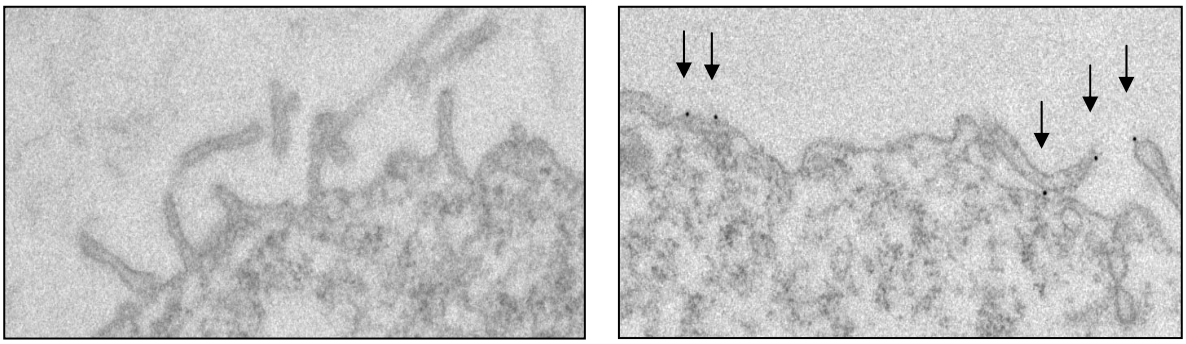


Fig 3.39: Optical micrographs showing stable insect cells expressing FHTevCB1(417)-YFP. Images were taken under oil with a phase contrast microscope. The YFP was excited with a 490-530 nm band pass filter.

Immunogold labeled electron-microscope images



Sf9 cells **Sf9 cells expressing**

Fig 3.40: Electron micrographs showing immuno-gold labeling of the FHTevCB1(417)YFP receptor. The stably expressing *Sf9* cells were pre-embedded with the mouse anti-flag M2 antibody. Goat anti-mouse antibody loaded with gold particles was used to detect the receptor. Sections of cells embedded in resin were imaged by electron microscope to detect the gold particles. Arrows indicate the gold labeled receptors. Wild type *Sf9* cells were used as negative control and didn't show any spots. Experiment was done by Dr. Winfried Haase.

Chapter III: The CB1 receptor-G protein interaction studies

The cannabinoid receptor exists in a pre-coupled form to the G proteins. The receptor "R" can exist in a "RG_{GDP}" (GDP bound) form or "RG_" (nucleotide lacking) form in addition to the free "R" form (Howlett, 2004). These forms of the receptor are considered to be responsible for the sequestration and constitutive activity of the receptor (see Introduction 1.6.31).The present experiments have been carried out to investigate and prove this pre-coupled form, in the heterologous insect cell expression system.

3.9 FRET to confirm the CB1 receptor-G_i complex

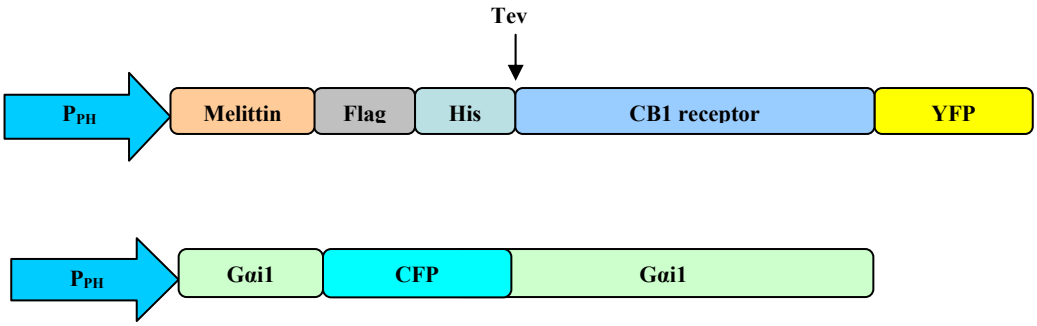


Fig 3.41: Gene constructs coding for the CB1 receptor and the Gi protein used for FRET experiments. P_{PH} stands for the polyhedrin promoter in the pVL1393 vector. YFP stands for the Yellow fluorescent protein and CFP stands for Cyan fluorescent protein. Other abbreviations are similar as in Fig 3.24. CFP protein was inserted in between the α-helical and the GTPase domains of the G protein.

Fluorescence energy transfer occurs when two chromophores are in close proximity. The CB1 receptor was obtained as a fusion protein withYFP and the G_i protein as a fusion protein with CFP. CFP and YFP form a good donor-receptor couple for the FRET experiments. Recombinant virus was produced using the constructs shown in Fig 3.41. *Sf9* cells co-expressing CB1-YFP, G_i-CFP and $\beta_1\gamma_5$ were imaged using a confocal microscope. Both proteins were found to be colocalized in the cell membrane. The fluorescence energy transfer between the proteins was confirmed by acceptor bleaching experiment (Fig 3.43). When the acceptor protein was bleached using a high energy laser, there was an increase in the donor fluorescence. This result confirms that both proteins are in close vicinity, which proves that the receptor and G protein are present in a precoupled form. The increase in the donor fluorescence was calculated for 10 cells, the average 7%. As a negative control CB1-CFP receptor and H1-YFP receptor were co-expressed and the experiment was repeated the

same way as above. There was an increase of less than 2%, which could be a result of lateral overlap of FRET partners. This value was considered as a background signal.

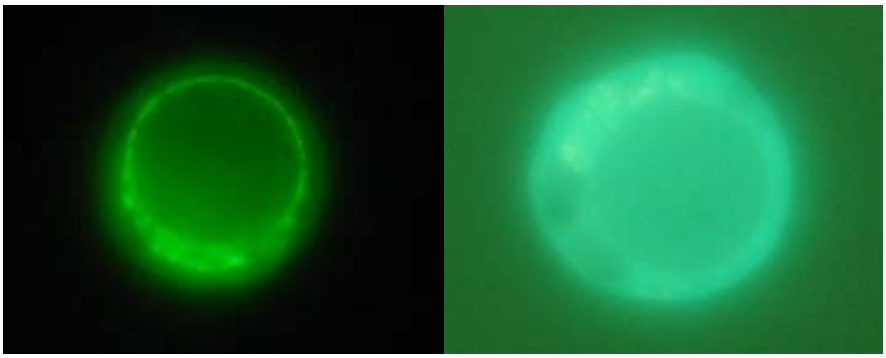


Fig 3.42: Fluorescence micrographs showing *Sf9* cells producing CB1-YFP receptor (left) and G*α*i1-CFP fusion protein. G protein produced in *Sf9* cells shows a uniform distribution throughout the cytosol. The CB1 receptor is located at the periphery and in the membranous region.

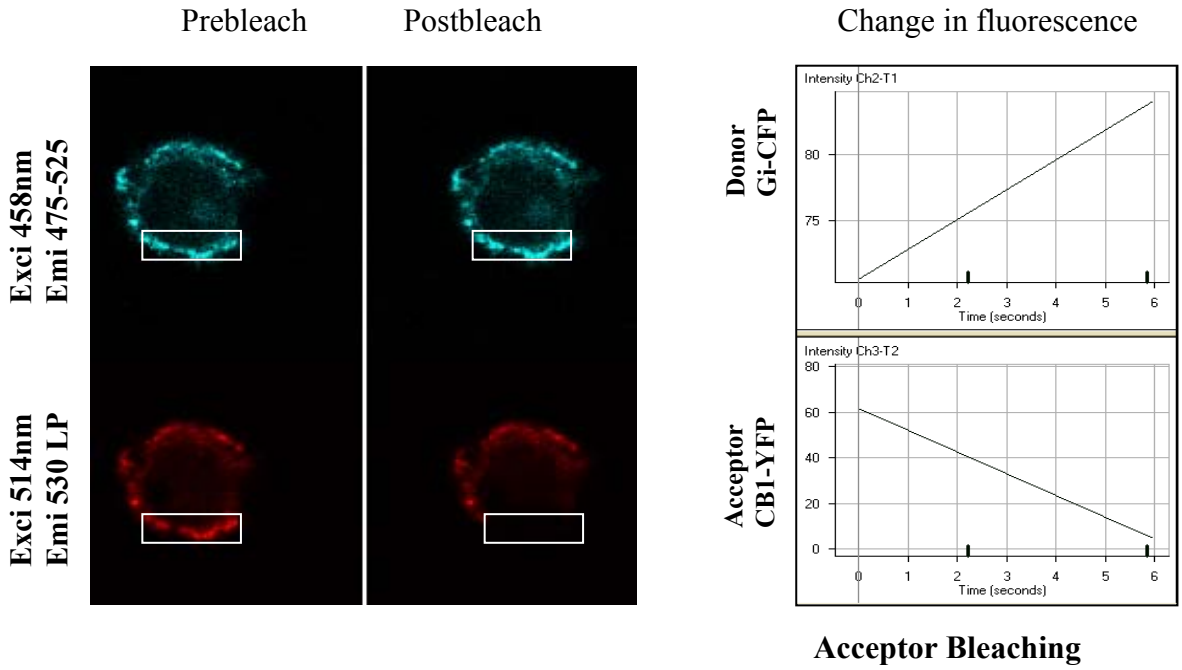


Fig 3.43: Confocal fluorescence micrographs showing FRET between CB1-YFP and G_i-CFP. The cyan colour in the confocal image represents the Gi protein. Red colour represents the CB1 receptor coupled to YFP. The white box represents the area bleached by the laser. Images in the first lane are prebleach images and the second lane are postbleach images. The graphs represent the increase in donor fluorescence and decrease in acceptor fluorescence during the bleaching period. 10 cells were imaged and the values obtained were averaged.

3.10 Constitutive activity of the CB1 receptor

When a receptor is constitutively active it leads to binding of GTP to G proteins even in the absence of agonist. *Sf9* cell membranes containing CB1 receptor (20 nM) and βγ dimer were mixed with the Gα proteins (100 nM) purified from *E. coli* and the change in the fluorescence due to the Bodipy GTPγS binding was monitored (Fig 3.44). Agonists or antagonists were not included in the reaction. *Sf9* cell membranes were used in the negative control. Addition of $G\alpha_i$ protein to the *Sf9* membranes increased already the Bodipy fluorescence for reasons unknown. But the increase obtained upon addition of cell membranes containing CB1 receptor was higher than that of native *Sf9* cell membranes.

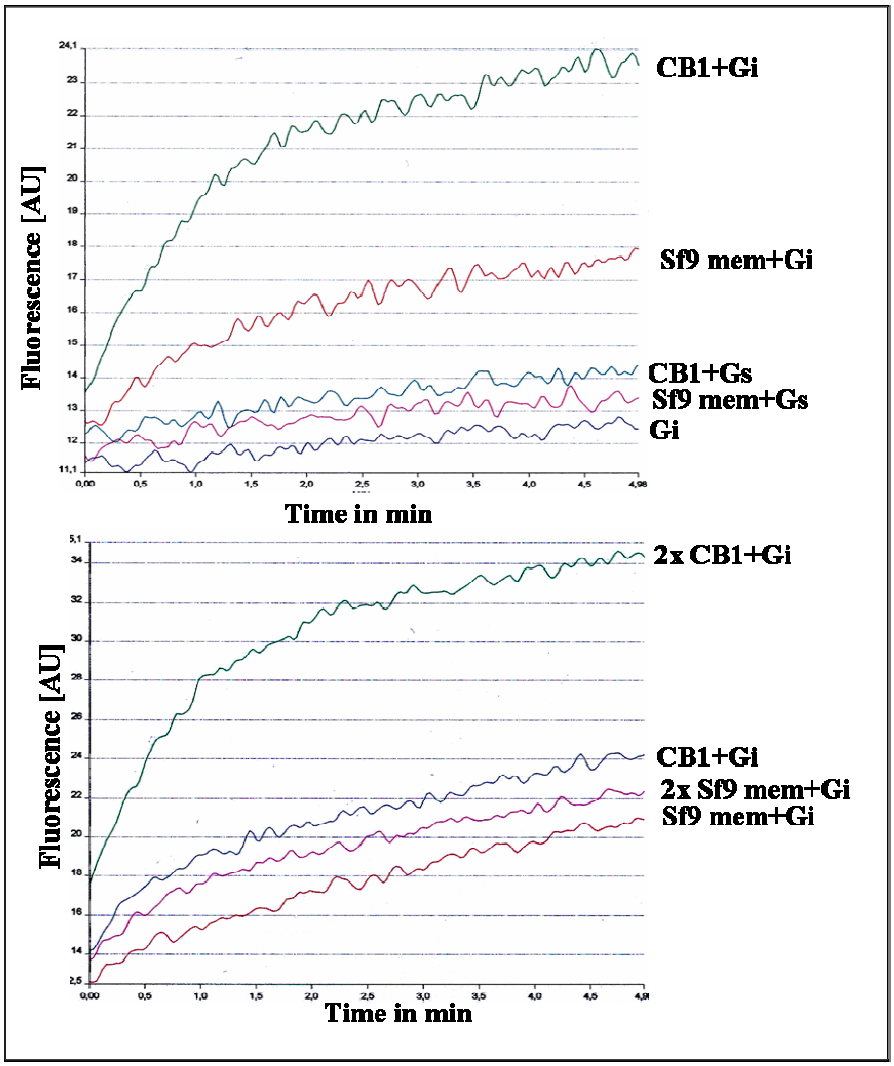


Fig 3.44: Fluorescence graphs showing the effect of receptor on GTP binding to the G protein. Above: Membranes with CB1 receptor enhance GTP binding to G_i protein but not to G_s. Receptor and G protein were used at 1:5 molar ratio. **Below: Membrane with CB1 receptor leads to a specific fluorescence enhancement.** Doubling of *Sf9* membrane in reaction doesn't change the GTP binding whereas CB1 membranes double the effect. Reactions were started by adding proteins to buffer containing Bodipy GTPγS.

An increase of the cell membrane concentration didn't increase the Bodipy fluorescence in the case of *Sf9* membranes but gave a clear additive effect with the membranes containing CB1 receptor. There was no significant change in GTP binding when $G\alpha_s$ protein was used instead of $G\alpha_i$ protein. The CB1 receptor doesn't bind to G_s under normal conditions.

3.11 GTPγS^{35} binding assay to study CB1 receptor-G_i interactions

Sf9 cell membranes containing heterotrimeric G proteins alone or together with the cannabinoid receptor were used for the radiolabeled GTPγS^{35} binding assay. The presence of all subunits in the cell membranes was confirmed by immunoblotting against each subunit. There was a clear increase in the GTPγS^{35} binding when the receptor was co-expressed along with the G proteins (Fig 3.45). This increase (~ 40% of the control) shows the constitutive activity of the receptor. Addition of the agonist WIN 55,212-2 increased the GTPγS^{35} binding to a lesser extent. Addition of the antagonist AM251 completely inhibited the GTP binding to the G proteins. These ligand dependent effects could not be seen in the membranes lacking the receptor (control membranes). A similar observation was reported by Glass *et al.* (1999), where cannabinoid receptors present in *Sf9* cell membranes and G proteins purified from brain were reconstituted.

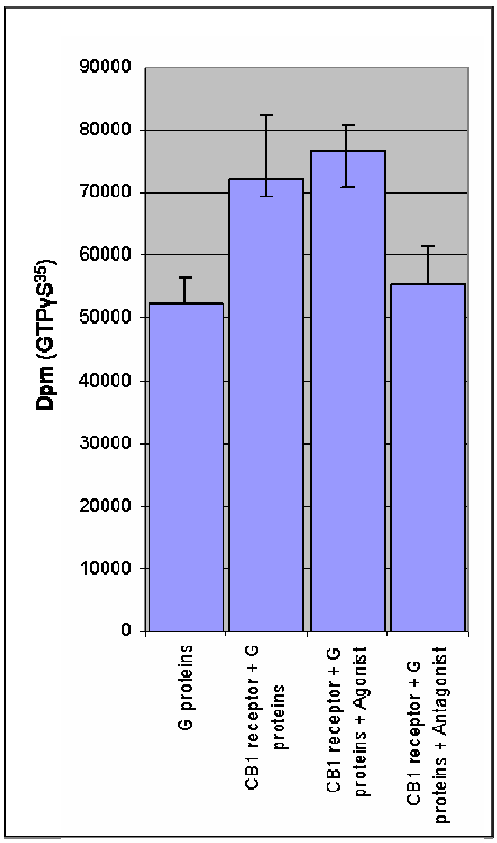


Fig 3.45: GTPγS^{35} binding to the G proteins. 30 µg of the Sf9 cell membrane expressing $G\alpha_{i1}\beta_1\gamma_2$ were used to check the background binding of GTP. 30 µg of membranes co-expressing CB1(417) receptor along with the G proteins were used to check the receptor induced increase in the GTP binding. 4µM agonist WIN 55,212-2 and antagonist 4 µM AM251 were used to see the ligand effect. Membranes were incubated at 30ºC for 1 hr. 4 nM radiolabeled GTPγS was used in the assay reactions. 10 µM GDP was included in all assay conditions. DPM is disintegrations per minute.

3.12 Co-immunoprecipitation of CB1 receptor-G protein complex

*Sf*9 cell membranes containing $G\alpha_{i1}\beta^1\gamma^2$ protein complex only or together with FHTCB1(417)StII were used for co-immunoprecipitation. The membranes containing only G proteins or receptor + G proteins together were solubilized using 1% decylmaltoside + 0.2% cholesterol hemisuccinate mixture for 1 hr. The clarified supernatant was incubated with anti-flag antibody agarose at 4˚C for 1 hr. The matrix was washed thrice with buffer containing 0.2% DM + 0.04% CHS (5:1). Both samples were incubated in SDS-Gel loading buffer for 15 min. The samples were analysed using SDS-PAGE and immunoblotted (Fig 3.46). The immunoblot with anti-flag antibody showed the CB1 receptor band at 45 Kda. An anti-his tag immunoblot showed the receptor band as well as the βγ dimer (γ has his tag on N-terminus) at ~34 Kda. The immunoprecipitated sample from the membrane containing only G proteins didn't show any specific band in either immunoblots. The immunoprecipitated sample from both membranes containing G proteins alone or receptor + G proteins together showed the Gα protein in the anti-G_{i1}immunoblot indicating a non-specific interaction of Gα protein with the matrix. But the signal obtained in the case of receptor + G proteins was higher than that of the G protein alone.

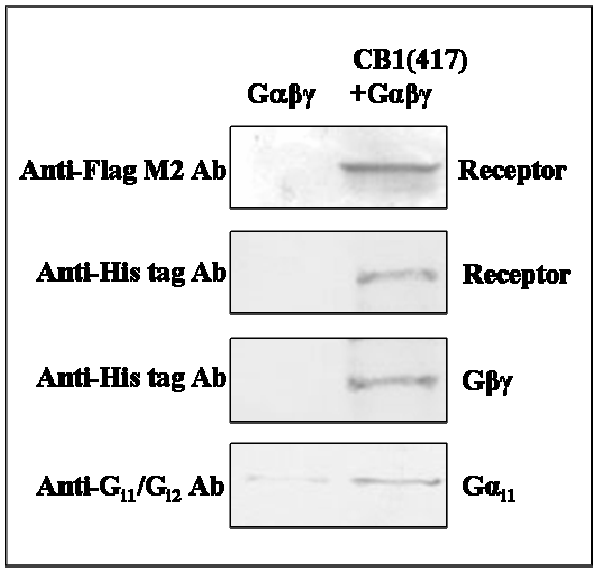


Fig 3.46 : Immunoblots showing co-immunoprecipitation of CB1 and $G\alpha_{i1}\beta^1\gamma^2$ complex: Sf9 cell membranes containing CB1-417 receptor and $G\alpha_{i1}\beta^1\gamma^2$ trimer complex was solubilised using mixture of DM and CHS. The complex was immunoprecipitated using anti-flag M2 antibody agarose matrix. The matrix was washed thrice and the bound protein was eluted by denaturation with SDS-Gel loading buffer. Lane 1= cell membrane solubilized contain only G protein trimer, Lane 2= cell membrane contains both receptor and G protein trimer. Antibodies used for identifying the different subunits of the complex are denoted on the left side of the image. The subunit that was identified is mentioned on the right side of the immunoblot image. Anti Gi1/Gi2 antibody immunoblot showed that Gα subunit has non-specific binding, to the matrix. But the intensity of Gα subunit was much higher when the receptor was present in the solubilizate.

4. Discussion..

4 DISCUSSION

4.1 Need to produce GPCR and G proteins

The aim of the project is to find out the possibility of using G proteins to co-crystallize with G Protein Coupled Receptors (GPCRs). GPCRs are membrane proteins and are difficult targets for structural determination by crystallography. The only known structure of a GPCR is that of bovine rhodopsin which is abundantly available in the retina (Palczewski *et al*., 2000). The amphipathic nature of the proteins renders them unstable in aqueous solutions and they need to be kept in a soluble form using the detergents. Most of the protein is covered with the detergent exposing little of its hydrophilic surface to make contacts during crystallization. The hydrophilic portion which is comprised of N and C-termini and the loop regions are highly flexible and may not be the suitable crystal contact regions. How ever this speculation might not be completely true. Most of these regions were assigned in the crystal structure of bovine rhodopsin, except a few cytosolic fragments which are supposed to be highly dynamic and interact with G proteins (Palczewski *et al*., 2000).

One possibility to overcome this problem is to increase the hydrophilic part of the protein which is more conformationally stable unlike the loop regions. The use of antibody fragments to increase the hydrophilic portion of the membrane proteins is a successful practice nowadays (reviewed by Hunte and Michel, 2002). Instead, if we can use physiologically interacting proteins like G proteins and arrestins for this purpose, the structure of the whole complex gives us valuable information about the functional interactions among them. There are several things we need to know before we think of co-crystallizing these proteins. Does the interaction make these proteins to form a stable complex or is this just a transient interaction? If they form a complex, how strong is it? Can

we have this complex *in vitro,* in a stable form for longer times needed for crystallization? Is it possible to express these proteins separately and mix them stoichiometrically to get a complex? Is it possible to express them together and purify the whole complex? To find an answer for these questions we need large amount of pure protein first of all.

4.2 G protein production

G proteins are the cytosolic counterparts of GPCR mediated signal transduction, which physically interact with the receptor at the membrane. G proteins are heterotrimers formed with one each of α, β and γ subunits. *E. coli* was used as a successful expression system for Gα protein production (Lee *et al*., 1994). Human Gα proteins (G_i, G_s, G_t) produced in *E. coli* were crystallized successfully (Noel *et al*., 1993; Coleman *et al*.,1994; Mou *et al*., 2006), in their ligand bound forms.

In the present work, 17 Gα subunits were cloned using Gateway cloning technology into pDEST14 vector under the T7 promoter. Gene sequences were analyzed by Rare codon calculator (RaCC) and found several rare amino acid codons in these human G proteins. So, BL21(DE3) Codon plus and Rosettagami(DE3)pLysS strains containing the t-RNAs for rare amino acid codons were used for protein production. $G\alpha_s$ family (except $G\alpha_{olf}$) and $G\alpha_{i/o}$ family members could be expressed in higher yields (> 5mg/l) compared to the other proteins. $G\alpha_q$ and $G\alpha_{12/13}$ family proteins could not be produced in *E. coli*, for reasons not clear. Gαq protein could be expressed in the *Pichia pastoris* expression system. This is a first attempt to produce G protein in *Pichia*. The protein was purified and crystallized. The initial crystals diffracted to 6.5 Å. Unexpectedly the protein production level has gone down drastically that it can not be purified anymore. This anomalous behaviour was observed in the lab for some other proteins as well. But the reasons are not clear yet. But recently $G\alpha_q$ (Tesmer *et al*., 2005) and $G\alpha_{13}$ (Chen *et al*., 2005) proteins have been expressed in insect cells in a chimeric form where the N-terminus of these proteins were exchanged with that of $G\alpha_{i1}$ protein. Both proteins were crystallized and structures were determined. This shows that the N-termini of these proteins are crucial in their production. May be the acylation is a requisite for the successful production of these proteins or presence of several positively charged residues present on the N-terminus of the protein reduces the protein production in prokaryotic expression system.

The production of the Gβ and Gγ subunits was not very successful in *E. coli*. Only two Gβ subunits could be expressed and none of the Gγ subunits. There are reports confirming that the Gβ and Gγ need to be expressed together to make a functional complex. It was found that, the Gβ co-expression is needed for the proper processing of the Gγ subunit and expression of the subunits individually makes them non-functional (Pronin *et al*., 1993). This might be the reason why they could not be expressed in *E. coli*. Another explanation would be the small size of Gγ subunit and the simple helical structure that makes it highly prone to degradation. The Gβγ dimer produced in *P. pastoris* could not form a trimer complex with Gα subunits tested by gel filtration. It was also observed in the present work that most of the Gβγ dimer produced was in the cytosolic portion than in the membrane. Several reports confirm that Gα proteins can be produced in any expression system but Gβγ should be obtained from higher eukaryotic system in order to make a functional trimer complex (Resh, 1999). This shows the significance of isoprenylation on the Gγ subunit. It is not yet clear if *P. pastoris* can make this modification to Gγ subunit.

4.3 Possibility of using G proteins for co-crystallization

GPCR is an integral membrane protein and needs detergent to keep it in solution. If G proteins are to be used together with it, we have to confirm their stability in detergent solution. The same was done and surprisingly not all Gα subunits retained the capacity to bind to GTP. The $G\alpha_s$ family members were highly susceptible to the detergent. Detergent above 1 CMC destroyed the G protein activity. This denaturation is not permanent but was reversible upon removal of detergent. This effect was the same with ionic and non-ionic detergents. The probable explanation is that the detergent is binding to a hydrophobic patch on the protein and thereby changing the conformation of the protein and thereby the binding pocket for GTP. On the other hand $G\alpha_{i/o}$ family proteins seemed to be active even at 50 times CMC (LM) of detergent. So the idea of using $G\alpha_s$ family members for the co-crystallization experiments might be improbable. $G\alpha_{i/o}$ family members are likely candidates for this purpose.

Is Gα subunit sufficient to bind to the receptor or does it need Gβγ as well. This question is still debatable. In a report on solubilized N-formyl peptide receptor / G protein interaction studies, it was reported that individual Gα or Gβγ subunits could not alter the

ligand binding to the receptor (Bennett *et al.*, 2001). The heterotrimer was needed. Whereas, in a recent paper on the rhodopsin / transducin interactions, it was reported that a native acylated Gα protein alone was capable of interacting with the receptor. But if Gα subunit lacks acylation, then it needs acylated Gβγ complex (Hermann *et al.*, 2006). So, with the present knowledge we have, Gα proteins produced in *E. coli* might not be sufficient for the purpose of co-crystallization.

What are the other probable alternatives for this purpose of co-crystallization? If it is difficult to produce subunits of the complex separately and make a functional complex *in vitro*, it might be possible to isolate the whole complex from the cell membranes. In the recent reports more and more GPCRs are found to be present in a complex with G proteins. If such a complex is not a mere transient complex, but stable, it can be isolated to use it for crystallization. But it is necessary to find out the ways to stabilize such a complex. The other potential proteins that can be used to co-crystallize with GPCRs are β-arrestins. β-arrestins bind to the activated and phosphorylated receptors and terminate the signal of the receptors (reviewed by Pierce and Lefkowitz, 2001). These proteins, unlike G proteins are few in number and so bind to many receptors. One more class of physiologically interacting proteins with GPCRs are the scaffold proteins, which link GPCRs with several effector molecules (Hall and Lefkowitz, 2002).

4.4 Why cannabinoid receptor?

The cannabinoid receptors bind to $G_{i/o}$ family members which are stable in detergent solution. An interesting feature of cannabinoid receptors is that they exhibit G protein sequestration and constitutive activity. By sequestration, cannabinoid receptors steal the $G\alpha_{i/o}$ proteins from a common pool making other GPCRs which use these G proteins non-functional (Vásquez, *et al*., 1999). This gives us an idea that one of the conformations of these receptors do exist in a complex with the G proteins which is not just a transient complex that forms upon activation by agonist. The next thing we should know is whether this conformation could be stabilized and maintained. The recently proposed mechanism of G protein sequestration by cannabinoid receptor 1 gives us a possible clue. According to this model, the receptor exists in two conformations: active and inactive. Both conformations also exist in a precoupled form to the G proteins. The active form in

complex with the G proteins is responsible for constitutive activity of the receptor. The inactive form precoupled to the G proteins can not be activated by agonists and is also responsible for the sequestration. Inverse agonists stabilize this inactive conformation and so boosts the equilibrium towards the inactive receptor bound to G proteins (IAR°G) (refer to the Fig: 1.15 in Introduction section). A recent report (Mukhopadhyay *et al*., 2005) gives the experimental proof for this hypothesis. It was shown by co-immunoprecipitation that CB1 receptor and G proteins exist in a precoupled form even in the absence of ligands. It was also shown that different ligands stabilize the different CB1/$G\alpha_i$ subtype complexes. These experiments were done using mammalian cells. If this CB1/$G\alpha_i$ complex can also be produced at high levels in heterologous expression system, it can be used for isolation and structural determination. This is one of the aims in this project, to see if this functional CB1/$G\alpha_i$ complex can be produced in the insect cell expression system, at higher levels.

4.5 Production and purification of cannabinoid receptors

Cannabinoid receptors were produced in different expression systems for functional and structural analysis. The initial trials of production of cannabinoid receptors for purification, was done by Grisshammer's group in 1997. They reported that the CB2 receptor could be produced to 38 pmol/mg but the CB1 receptor could not be obtained. The reason was the extensive degradation observed for the cannabinoid receptor 1 (Calandra *et al*., 1997). Following this work several groups have produced and purified the functional CB2 receptor in *E. coli* and purified to 85% (Yeliseev *et al.*, 2005).

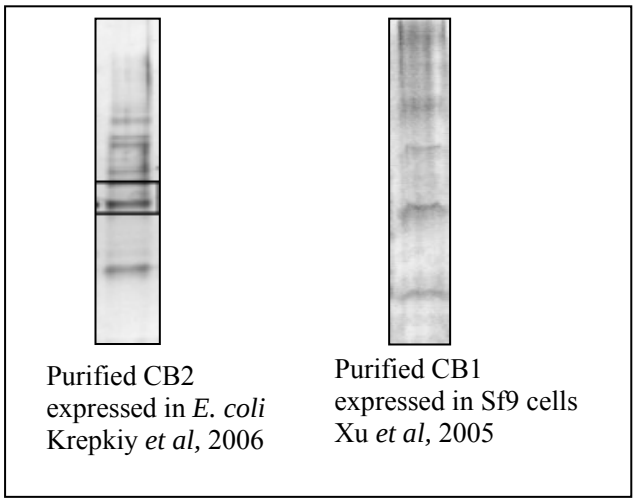


Fig 4.1: Purified cannabinoid receptors analyzed by SDS-PAGE. Images taken from the publications of Krepkiy et al.,2006 and Xu et al., 2005.

Song's group tried to express both the receptors in *P. pastoris* expression system. (Kim *et al*., 2005). The results show that the yields of the proteins were sufficient to analyze by mass spectroscopy only. The reason for the poor protein yield was extensive washing of Ni-NTA matrix. Extensive washing was needed to improve the protein purity. The baculovirus mediated insect cell expression system was also explored by Makriyannis's group. They faced the same problem of purification and low yield. Harsh washing steps such as washing with 8 M Urea were used in these purification experiments. So these results show that not a single successful protein purification system has been established yet for cannabinoid receptors. The purification of large amounts of functional receptors needed for structural studies is still to be explored, which is one of the aims of the present work.

4.5.1 Production of the cannabinoid receptor 2 in *P. pastoris*

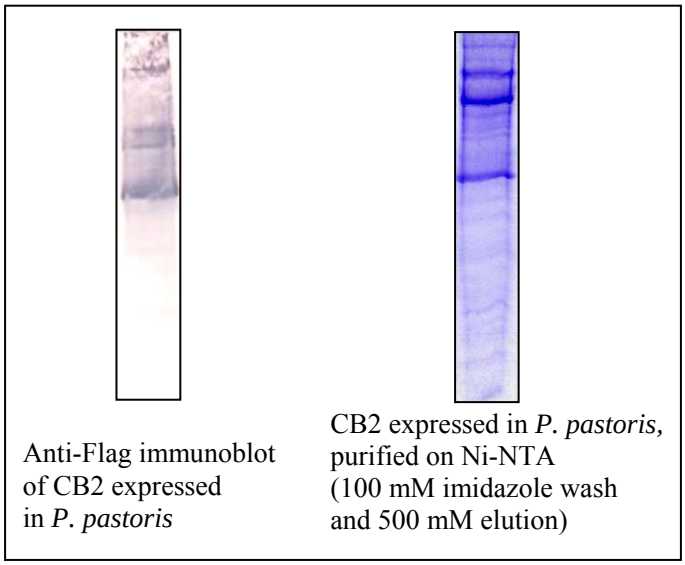


Fig 4.2: Immunoblot and Coomassie stained SDS-PAGE gel showing the purified CB2 receptor. The band pattern on the SDS-PAGE shows high impurity levels.

The protein produced was functional with respect to ligand binding. The western blotting analysis showed that there were several bands corresponding to the receptor. A smear was observed at the higher molecular weight range showing extensive aggregation of the recombinant protein produced. The protein obtained from Ni-NTA purification was impure. Washing had to be done with high (100 mM) imidazole concentration, generally used to elute the protein, to improve the purification. But there were still several high molecular weight bands which don't correspond to the receptor. These results were similar to those published by Feng *et al*. (2002) during the same period. Purification was not better

even after using several other additives like glycerol, sucrose, urea to the buffers during washing. The second purification using the bio-tag also did not improve the purity. Most of the protein was found bound to the matrix resulting in extremely low recovery of the receptor. This might be because of the presence of irreversible high affinity sites on the matrix for biotin. The gel filtration profile showed that the protein was aggregated. This means that the receptor was not homogenously produced in *Pichia*.

4.5.2 Production of cannabinoid receptors in insect cells

Several groups have tried to express cannabinoid receptors in insect cells. Purification attempts were also made for both the subtypes of receptors. The results show that the purification yields were very low and impure. Special note could be given to CB1 receptor (Xu, 2005) where the authors report that several receptor bands were observed after purification. All these bands were analyzed by mass spectroscopy. All the bands correspond to the CB1 receptor. But in the lower band they couldn't detect the N-terminal peptide, which might mean that this is a degradation product. A dimer corresponding to this truncated protein was also observed. In the present work I tried to purify the CB1 receptor using different tags (Fig 4.1). Several constructs were made to produce recombinant receptors fused to poly-histidine tag, Strep-tagII and Biotag at the termini. Worth mentioning is the truncated CB1 receptor where the C-terminal 54 amino acids were removed, to make a CB1 receptor with 417 amino acids. The importance of this construct is that the C-terminal 17 (401-417) amino acids are responsible for G protein activation and sequestration. According to Nie *et.al* the truncated version enhanced both the constitutive activity and the ability of the receptor to sequester G proteins. This will be more useful for co-crystallization. All gene constructs produced the recombinant proteins in insect cells. The receptor produced, gave two bands on the immunoblots, major monomeric band and a higher oligomeric (~ 160-175 KDa) band probably tetrameric. This high molecular weight band was also observed by earlier investigators in brain tissue and other heterologous expression systems as well (Wager-Miller *et al*., 2002). The protein produced was glycosylated as determined by the tunicamycin experiment.

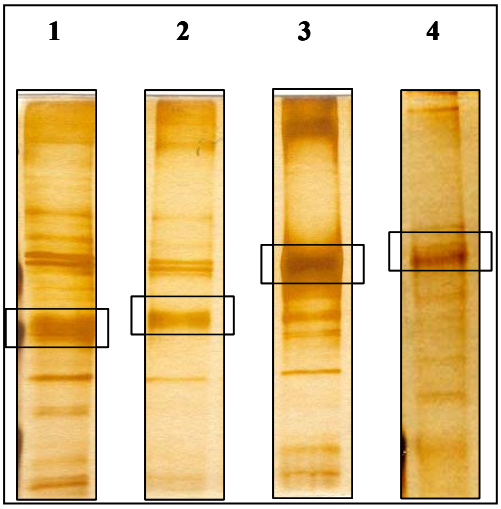


Fig 4.3: CB1(417) receptor purification. 1. Ni-Tev-Ni purification of CB1(417)StII 2. Ni-NTA and Streptactin purification 3. Ni-NTA and Monomeric avidin purification of CB1(417)Bio 4.His-Select purification of CB1(417)Bio

A two step affinity purification was followed for all the recombinant proteins produced. Protein purified from Ni-NTA column was highly impure. Ni-Tev-Ni strategy didn't result in any betterment in purification. Digestion with the Tev protease was complete but the purity was not better. This might be because the impurities interact with the receptor or with the detergent micelle. Use of Strep-tagII improved the purity of the receptor. But the protein yield was very poor because of the loss of receptor in the flow through and wash. Very little protein specifically bound to the matrix which could be eluted. The reason could be an inaccessible strep-tagII or a conformation of the C-terminal tail reducing the affinity of the tag to the Streptactin matrix. Experience with the biotag was contrary. Monomeric avidin matrix was used to purify the receptor using the biotag. As experienced in many cases the receptor seemed to be sticking to the matrix which could not be specifically eluted. This in-turn reduced the recovery drastically. So neither Strep-tagII nor biotag were helpful for the receptor purification.

Another possibility to improve the purity was optimizing the IMAC purification. Different IMAC resins were tested. Out of them His-Select from Sigma gave better purity. This matrix has less Ni density compared to Ni-NTA from Qiagen, which might have lead to lesser non-specific binding and thence better purity. Another Ni-IDA based matrix from Biorad (Profinity IMAC) resulted in loss of protein during loading itself because of week binding to the matrix. Protein obtained from His-Select purification and Ni-NTA/Streptactin was better than other methods of purification, but the yield was not better. The gel filtration of these samples did not give a symmetric peak, because the protein is not homogenous. From the retention volume of the peaks (Fig 3.38) it can be said that protein

is present in oligomeric forms. The N-terminal truncated receptor (Fig 3.37-C) lacking N-terminal tags might be the reason for lower yields in all the purification strategies. All the receptor that is present in the membrane is not captured by IMAC matrix. These results show that a lot of effort is still needed to improve the yield and purity of the protein. One important aspect to be thought of at this point is why there are so many impurities during the receptor purification.

4.6 Reasons for the impurity

There are several possible reasons for the high impurity levels for this receptor purification. One reason could be the degradation of the receptor itself as was observed by Xu *et al,* 2005. These N-terminal truncated forms could also form dimers or oligomers with the full length receptor and co-purify with it. Andersson *et al*, 2003 reported that compared to other class A receptors, CB1 has an exceptionally long N-terminal domain of 116 amino acids, which causes problems during protein targeting. They report that there is no clear signal sequence at the N-terminus and so it cannot be efficiently translocated across the ER membrane, causing the rapid degradation of CB1 receptor. They propose that receptor trafficking might be mediated by chaperones. It might also be possible that these chaperones might be some of the impurities observed.

A more probable reason for impurities could be that these are the proteins interacting with the receptor. Both, the yeast *Saccharomyces* and insect cells (*Sf9*, High Five Knight, 2004) possess G proteins which are homologs of mammalian G_q and G_i proteins. Cannabinoid receptors are known to exist in a precoupled form with the G proteins. These endogenous G proteins from the insect cells or yeast (not known for *Pichia*) might constitute some of the impurities. Another possible reason is that the impurities are other endogenous GPCRs which might form dimers or oligomers with the CB1 receptor. In *Drosophila melanogaster* there are about 200 genes coding for GPCRs. Even though there are no cannabinoid receptors in insects (McPartland, 2001), there are sequences which are homologous to other mammalian receptors like dopamine receptors (Brody, 2000). Dopamine receptors are known to form dimers with cannabinoid receptors. Though it is not clear about *Pichia*, Sf9 cells might have some GPCRs similar to dopamine receptor. So the endogenous GPCRs could also form dimers with the heterologously expressed CB1

receptor and co-purify with it. But these reasons don't sound convincing for the impurities obtained during CB2 receptor purification, which was expressed in *E. coli* (Krepkiy *et al,* 2006). Several reports have shown that amino terminus is responsible for the rapid degradation of the CB1 receptor (Nordstroem *et al*, 2006).

What more could be done to solve the problem of purification is a question to be thought of at this point. One probable way could be the expression of an amino terminal truncated receptor, in yeast or insect cells. This approach can solve the problem associated with truncation of the receptor. But how many amino acids can be removed from the N-terminus, without destroying the function of the receptor has to be investigated. The CB1 receptor and CB2 receptor share a 70% identity in the transmembrane regions. The CB2 receptor has been successfully produced and purified where as the purification of CB1 receptor is still a challenge. So another idea is to try to produce, in *Pichia pastoris* or insect cells, a chimeric protein between the amino terminus of the CB2 and the transmembrane regions of the CB1 receptor. Since it is more identical to CB2 receptor, it could be possible to purify this chimeric receptor.

4.7 Ligand binding properties of the CB1 receptor

Expression levels: Radioligand binding experiments were performed both with agonists and antagonists of cannabinoid receptor 1. Cannabinoid ligands are highly lipophilic with high lipid partition coefficient. They are almost insoluble or feebly soluble in water. This is why the non-specific binding is too high up to 80%. At least 1% BSA had to be included in the binding assay buffer to reduce the non-specific binding. BSA acts as a natural carrier for lipophilic molecules in the blood. The saturation binding assays gave Kd values comparable to the literature values (0.6 nM-7 nM). The Kd value for the full length CB1 receptor and C-terminal truncated CB1(417) is between 2.0 to 3.6 nM. The Bmax values for the CB1StrepII and CB1(417)StrepII were 40 and 53 pmol/mg membrane protein respectively. This expression level is almost two fold higher than the levels reported for the CB1 receptor (24 pmol/mg in Xu *et al*., 2005). The agonist saturation binding of CB1StrepII gave a Bmax 5 times lower than that of antagonist value. This shows that most of the receptor produced was in an inactive R conformation, which has high affinity for the antagonist but does not bind agonist.

Salt and Mg^{2+} effect: High sodium chloride concentrations reduced the agonist binding to the receptor. But there was no effect on the antagonist binding. The Kd of the agonist CP 55,940 to the receptor was almost double in the presence of 1M NaCl. The Bmax was also reduced to 60%. This is consistent with the earlier discoveries that Na^{+} reduces the agonist binding to the Gi coupled GPCRs (Swaminathan *et al.,* 2003). Mg^{2+} was necessary for agonist binding but not for antagonist binding. Mg^{2+} might have an allosteric effect on agonist binding. Zn^{2+} was shown to have a similar effect on the β-adrenergic receptor (Swaminathan *et al.,* 2003).

Detergent effect: Detergents like OG, LDAO, Foscholines at 1.5 CMC completely hindered the ligand binding even though the exposure time of cell membranes and the detergents was only a few seconds normally not sufficient for solubilization. This could be because the ligand is entrapped in the micelle and not available for receptor binding. Laurylmaltoside and decylmaltoside didn't reduce the ligand binding in this pre-screening and hence these detergents were selected to use in protein purification.

Ligand binding could not be detected in the solubilized or purified cannabinoid receptor. Several techniques like glass fiber filters, activated charcoal, calcium phosphate, gel filtration have been used to investigate the ligand binding. There was no specific binding. The high non-specific binding was the reason. Cannabinoid ligands show high non-specific binding because of their high lipid partition coefficient. Glass fiber filter assay is not a reliable technique because the solubilized receptor can pass through the filter pores. Gel filtration on Sephadex columns was also not reproducible because BSA used in the binding and wash buffers bind to cannabinoid ligands and elutes at the same void volume where the receptor is expected. There by giving high back ground binding. Water soluble ligands could help to solve this problem of high non-specific binding. But there is no such radiolabeled ligand commercially available.

DTT effect: DTT reduced the agonist binding to the receptor but not antagonist binding. The receptor retained the agonist binding capacity even till 10 mM DTT concentration where a disulphide bond doesn't exist. This shows that a disulphide bond might not be present in the receptor or not be important for ligand binding. This could be

true because in the predicted secondary structure of the receptor there is no cysteine in the first extracellular loop. This cysteine is the one which forms a disulphide bond with the cysteine in the second extracellular loop. Shire *et al.* in their work, to find out the structural features that are responsible for the ligand binding, proposed that cysteines in the second extracellular loop might directly play a role in ligand binding (Shire *et al*., 1996).

Ligands in the culture: Ligands were added to the culture to see if it is possible to produce the receptor in one confirmation. Presence of the agonist WIN 55,212-2 in the cell culture media didn't affect binding of the hot agonist CP55940 to the receptor. But when the antagonist AM251 was added to the culture, these cells or membranes didn't show agonist binding anymore. The possible explanation for this observation could be that the antagonist in the culture favours the inactive conformation "R" instead of active "R*" and so agonist cannot bind to the receptor anymore. So it might be possible that we can lock one particular conformation of the receptor by adding high affinity antagonist to the culture. This would be of help to get a conformational homogeneity.

4.8 The CB1 receptor-G*α*i precoupled complex in the cells

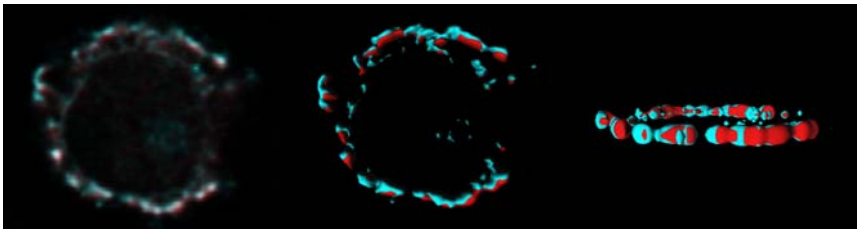


Fig 4.4: Confocal images showing the colocalized receptor and Gi protein. The blue regions represent the Gi-CFP and red regions represent the CB1-YFP.

Several investigations have shown that the CB1 receptor exists in a precoupled form with the G proteins. Two different precoupled forms can exist according to the ternary complex model. One is the inactive "RG_{GDP}" and other is active "R^*G_{GTP}" form. Nie *et.al* proved this by the patch clamp technique showing the constitutive activity of the CB1 receptor expressed in the nerve cells. "R^*G_{GTP}" form is responsible for the constitutive activity of the receptor. Present experiment showed that the existence of a precoupled form of recombinant proteins in a heterologous expression system. Cannabinoid receptor fused to

YFP, G_i protein fused to CFP and βγ were coexpressed in *Sf9* cells. Confocal microscopy was used to see the receptor-G protein precoupled form by FRET. When the acceptor was bleached there was an increase in the donor fluorescence upto 7% (average of 10 cells with min 4% and max 15% increase). This happens only when the donor and the acceptor molecules are in close contact and involved in FRET. As a negative control the histamine H1 receptor fused to YFP and the CB1 receptor fused to CFP were coexpressed and the experiment was conducted in the same way. There was a donor fluorescence increase of less than 2%, which might be because of the lateral overlapping of the molecules present in the membrane. This value was considered negative. A recent report confirms the existence of this kind of precoupled receptor-G protein complexes. Nobles *et al.,* 2005 showed that the muscarinic receptor (M4), the α adrenergic receptor (α2A), the adenosine receptor (A1), the dopamine receptor (D2) do exist in precoupled form with their corresponding G proteins. These investigations were also carried out using the FRET technique.

Cannabinoid receptor was shown to exhibit constitutive activity in the neuronal cells using patch clamp technique (Vasquez *et al.*, 1999). To investigate the constitutive activity of the receptor in a reconstituted system fluorescent GTP binding assay was performed. *Sf9* cell membranes expressing the CB1 receptor were mixed with the G proteins expressed in *E. coli* and purified. If the receptor shows the constitutive activity there should be an increase in the GTP binding without the addition of any agonist. G_s protein which doesn't couple to the CB1 receptor didn't show any enhancements in the GTP binding with or without the addition of CB1 receptor containing membrane. But G_i protein showed an additive increase of BODIPY GTPγS with increasing membrane concentration. No ligand was included in the reactions. These results prove that the receptor couples to the Gi protein even without the activation of agonist and exhibits basal activity.

A similar experiment was performed with the radioactive GTPγS ligand. It was observed that there was a significant increase in the GTP binding when CB1 receptor was co-expressed along with the G protein trimer. No ligand was included in this experiment. Agonist further increased the GTP binding but to a lesser extent, which could be because of the presence of receptor in an inactive conformation not recognized by agonist, as seen in saturation binding assay of CB1 receptor with agonist CP-55,940 (Table 3.2). Antagonist AM251 inhibited the receptor dependent increase in the GTP binding. These results are

similar to those observed by Glass *et al.*, 1999. They used a reconstituted system containing Sf9 membranes with CB1 receptor and G proteins purified from bovine brain. But from the present experiment it is evident that the cannabinoid receptor/Gi protein coexpressed in our heterologous expression system exhibits constitutive activity.

Co-immunoprecipitation of detergent (DM) solubilized FHTCB1(417)StII receptor and $G_{i1}\beta^1\gamma^2$ proteins using Anti-Flag M2 antibody agarose matrix proved that the receptor / G protein exist together and can be co-precipitated. No ligands were included in these experiments. Still both the partners were found to be coupled together. It is necessary to see if addition of ligands stabilize this complex as was seen in neuroblastoma cells in Mukhopadhyay *et al*., 2005. Further experiments are needed to see if this complex can be purified in a functional and homogenous form to use it for crystallization.

4.9 Conclusion:

The aim of the project was to investigate the possibility of using Gα proteins to co-crystallize with G protein-coupled receptors, several recombinant human Gα protein subunits had been successfully produced in the *E. coli* expression system. Some of these proteins were purified to homogeneity. Detergent stability of the G proteins was tested and found that not all G proteins were stable in detergent solutions. More and more reports are stressing on the necessity of a functional G protein trimer to form a GPCR-G protein complex, which is not possible in *E .coli*. It is more probable to produce the whole receptor-G protein complex in a higher eukaryotic expression system and use it for crystallization. For this purpose functional cannabinoid receptor 1 was produced alone and together with G proteins in the insect cell expression system. Extensive work on production of the cannabinoid receptor 1 resulted in levels almost double than those reported by other groups. Despite several difficulties in receptor purification, small scale purification had been established for the CB1 receptor. It was also proved that the cannabinoid receptor 1 and $G_{i1}\beta^1\gamma^2$ proteins form a functional complex in the insect cells. It was observed by FRET experiments that the receptor and G proteins are precoupled. This precoupled complex was confirmed by GTP binding experiments. It was also proved that the complex can be solubilized and co-immunoprecipitated. Further efforts are needed to purify the receptor or receptor/ G protein complex in higher yields needed for crystallization.

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Appendix..

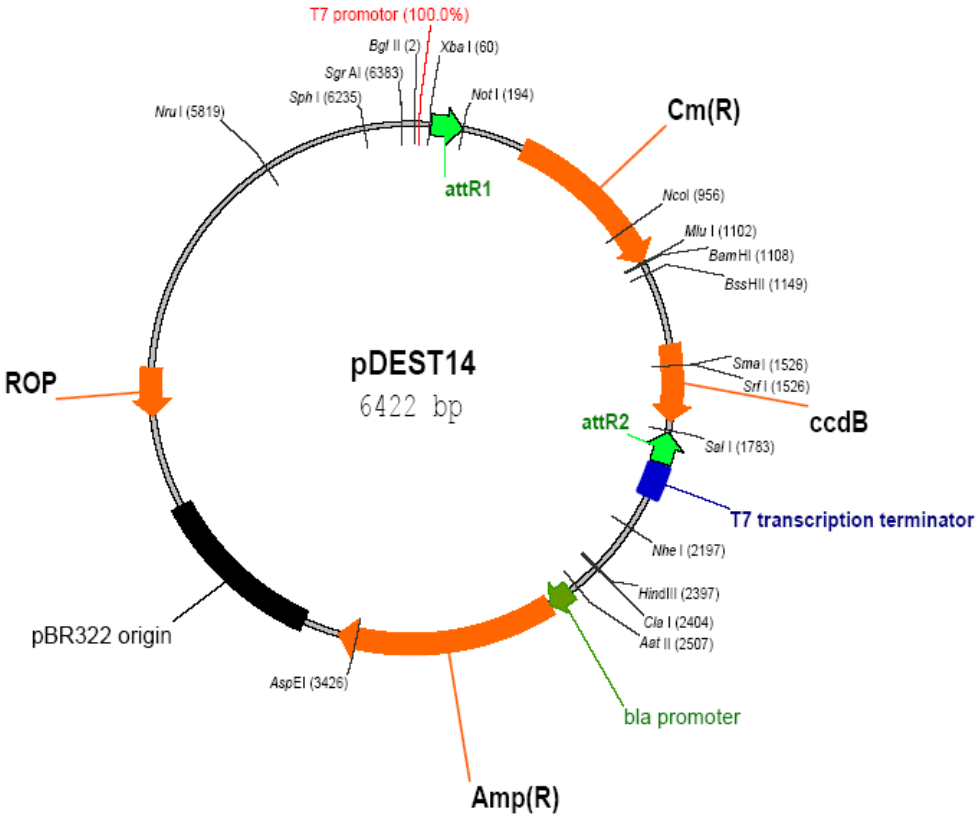
A1. <u>pDEST14 vector:</u> Expression vector used in GATEWAY® cloning technology

Resistance: Ampicillin and Chloramphenicol antibiotic resistance

Promoter: T7 promoter

E. coli Origin of replication: pBR322

Recombination: *att*R1 and *att*R2



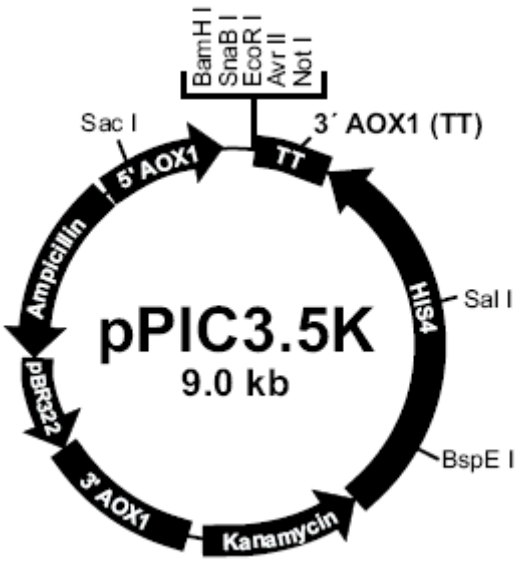
A2. <u>pPIC3.5K vector:</u> Vector for soluble protein expression in *P. pastoris*

Resistance: Ampicillin and Kanamycin

Selection in Pichia: His4 (gene)

Promoter: Alcohol Oxidase promoter (AOX1)

E. coli Origin of replication: pBR322



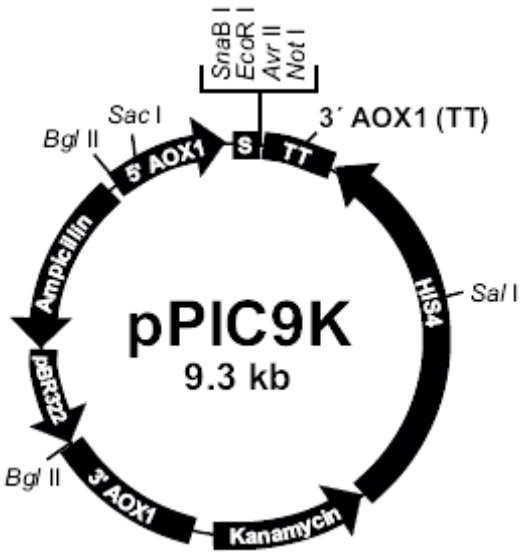
A3. <u>pPIC9K vector:</u> Vector for membrane protein expression in *P. pastoris*

Resistance: Ampicillin and Kanamycin

Selection in Pichia: His4 (gene)

Promoter: Alcohol Oxidase promoter (AOX1)

E. coli Origin of replication: pBR322

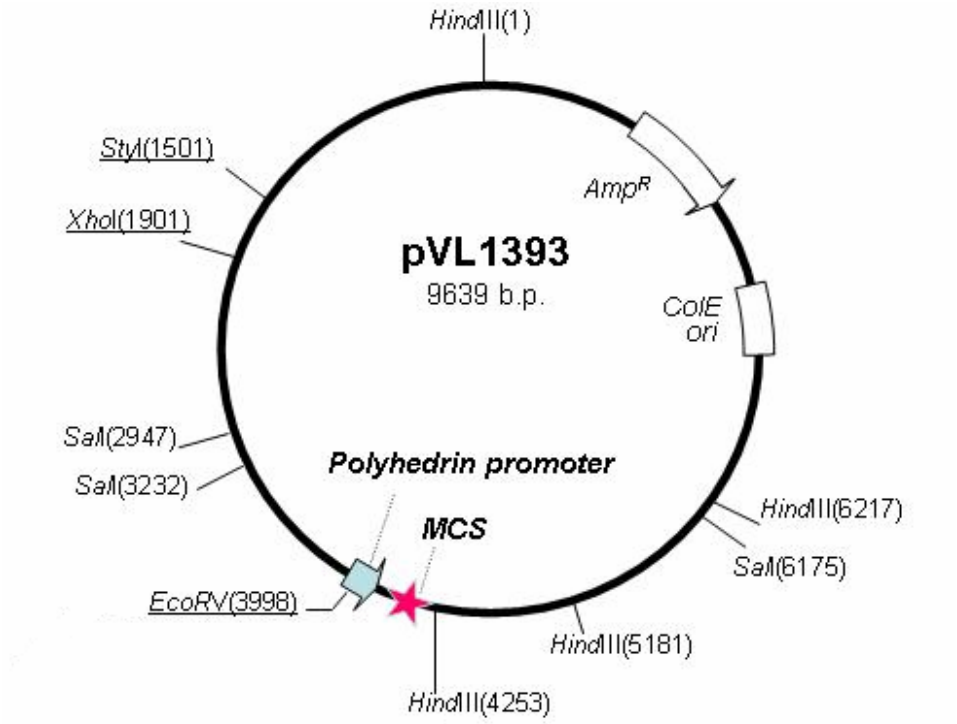


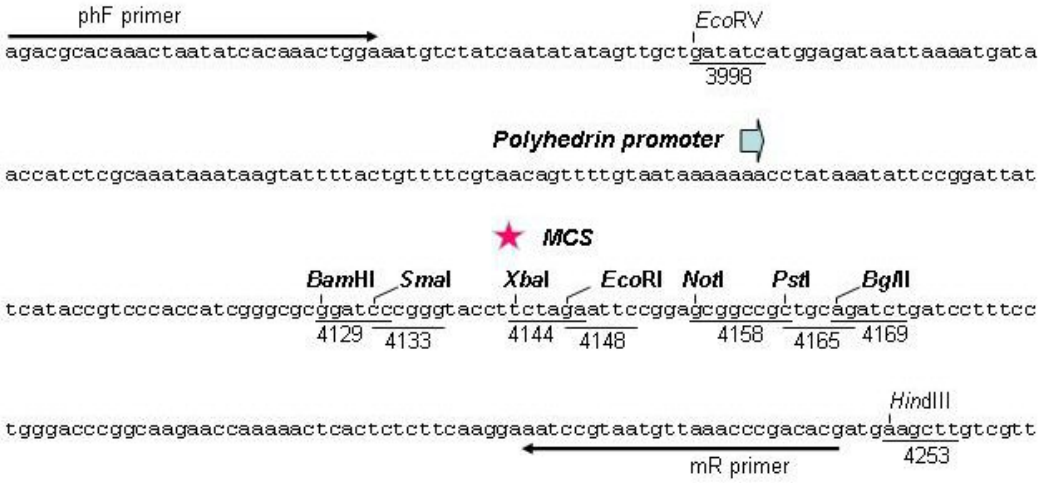
A4. <u>pVL1393 transfer vector:</u> Vector to transfer the foreign gene into baculovirus

Resistance: Ampicillin

Promoter: Polyhedrin promoter

E. coli Origin of replication: ColE





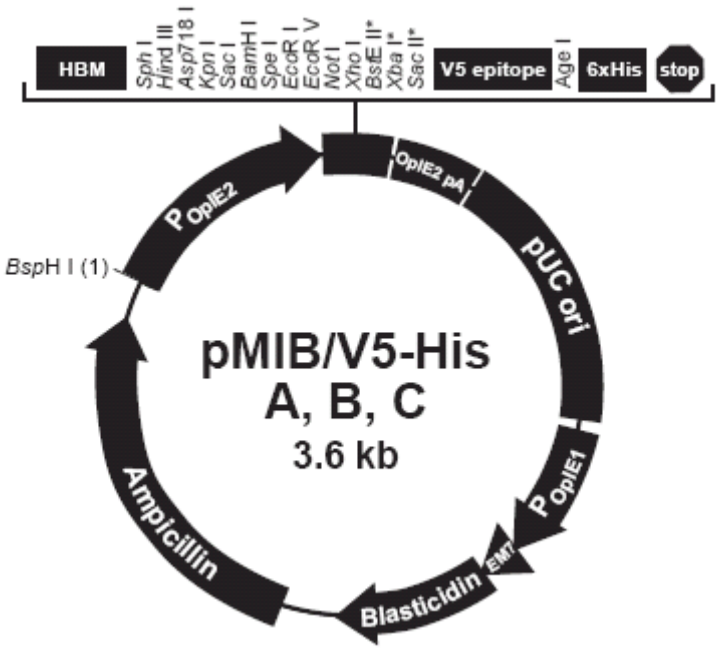
A5. <u>pMIB/V5-His:</u> Vector for generation of stable (*Sf9, Sf21, H5*) insect cell line expressing membrane proteins.

Promoter: pOpIE2 (from *Orgyia pseudotsugata*)

Signal sequence: Honey bee melittin signal sequence

E. coli Ori of replication: pUC

Antibiotic resistance: Ampicillin and Blasticidin



A6. CB1&CB2 protein sequence alignment

CB1: Human Cannabinoid receptor 1
CB2: Human Cannabinoid receptor 2

Identity: (31.9%)
Similarity: (43.9%)

```
CB1         1 MKSILDGLADTTFRTITTDLLYVGSNDIQYEDIKGDMASKLGYFPQKFPL     50

CB2         1                                                       0

CB1        51 TSFRGSPFQEKMTAGDNPQLVPADQVNITEFYNKSLSSFKENEENIQCGE    100
                                .::..:||..|.|......|
CB2         1                    MEECWVTEIANGSKDGLDSN--------     20

                                          TM1
CB1       101 NFMDIECFMVLNPSQQLAIAVLSLTLGTFTVLENLLVLCVILHSRSLRCR    150
                 .::.:|:|:..|:.|:|||...||..:.|||:.||.:||.|..||.:
CB2        21 ---PMKDYMILSGPQKTAVAVLCTLLGLLSALENVAVLYLILSSHQLRRK     67

                        TM2
CB1       151 PSYHFIGSLAVADLLGSVIFVYSFIDFHVFHRKDSRNVFLFKLGGVTASF    200
              |||.||||||.||.|.||:|..||::|||||..||:.|||.|:|.||.:|
CB2        68 PSYLFIGSLAGADFLASVVFACSFVNFHVFHGVDSKAVFLLKIGSVTMTF    117

               TM3                                        TM4
CB1       201 TASVGSLFLTAIDRYISIHRPLAYKRIVTRPKAVVAFCLMWTIAIVIAVL    250
              |||||||.|||||||:.:..|.:||.::||.:|:|...:||.::.:::.|
CB2       118 TASVGSLLLTAIDRYLCLRYPPSYKALLTRGRALVTLGIMWVLSALVSYL    167

                                                  TM5
CB1       251 PLLGWNCEKLQSVCSDIFPHIDETYLMFWIGVTSVLLLFIVYAYMYILWK    300
              ||:||.|  ....||::||.|...||:.|:...:.|...|:|.|.::|||
CB2       168 PLMGWTC--CPRPCSELFPLIPNDYLLSWLLFIAFLFSGIIYTYGHVLWK    215

CB1       301 AHSHAVRMIQRGTQKSIIIHTSEDGKVQVTRPDQARM--DIRLAKTLVLI    348
              ||.|..         |:..|  :|.:|    |..|||  |:||||||.|:
CB2       216 AHQHVA---------SLSGH--QDRQV----PGMARMRLDVRLAKTLGLV    250

                   TM6                                    TM7
CB1       349 LVVLIICWGPLLAIMVYDVFGKMNKLIKTVFAFCSMLCLLNSTVNPIIYA    398
              |.||:|||.|:||:|.:.:...::..:|..||||||||:||.|||:|||
CB2       251 LAVLLICWFPVLALMAHSLATTLSDQVKKAFAFCSMLCLINSMVNPVIYA    300

CB1       399 LRSKDLR----------------------HAFRSMFPSCEG----TAQP    421
              |||.:::|                      .|.||.....|.    |..|
CB2       301 LRSGEIRSSAHHCLAHWKKCVRGLGSEAKEEAPRSSVTETEADGKITPWP    350

CB1       422 LDNSMGDSDCLHKHANNAASVHRAAESCIKSTVKIAKVTMSVSTDTSAEA    471
              ....:..|||
CB2       351 DSRDLDLSDC                                          360

CB1       472 L                                                   472
```

| = identical amino acid, **:** =similar amino acid, **.** =dissimilar amino acid, TM = transmembrane helix

A.7 Primers:

CB1 receptor primers for baculovirus expression system

CB1FBV

5’ GC ***G GAT CC*** G ACC ATG GCG AAG TCG ATC CTA GAT GGC 3’

CB1RBV

NotI

5’ GAA T ***GC GGC CGC*** TCA CTT TTC GAA TTG AGG GTG CGA CCA

EcoRI

GAA TTC AGC CTC GGC AGA CGT GTC TGT GGA 3’

CB2 receptor primers for Baculovirus expression system

CB2Forward

5’ GC ***G GAT CC*** G ACC ATG GAG GAA TGC TGG GTG ACA 3’

CB2Reverse

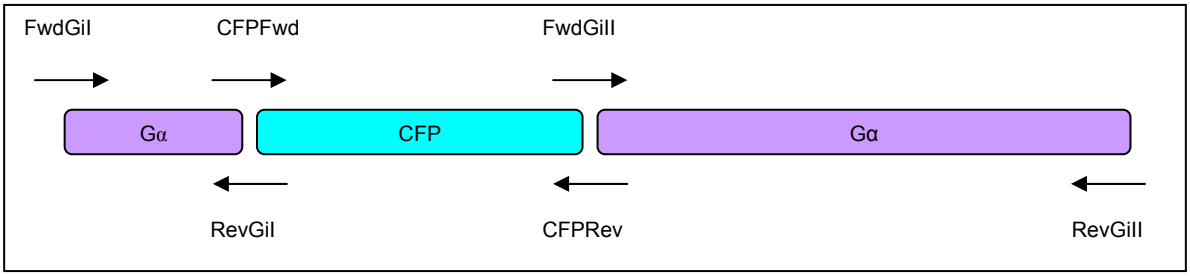
5’ CCA ***GAA TTC*** GCA ATC AGA GAG GTC TAG ATC 3’

CB1 receptor truncations

CB1-417Rev: CB1 C-terminal 55 amino acids truncation

5’ CCA ***GAA TTC*** GCC TTC ACA AGA GGG AAA CAT 3’

A.8 Gαi1-CFP fusion protein



PCRIGi:

FwdGiI: 5' GC ***GGA TCC*** ACC ATG GGC TGC ACG CTG AGC 3'
BamHI

RevGiI: 5' TTC ***TCT AGA*** CA***C CAT GG***T TGA ATA ACC AGC TTC ATG 3'
XbaI NcoI

PCRIIGi:

FwdGiII: 5' A***CC ATG G***TG ***TCT AGA*** GAA GAG GAG TGT AAA CAA 3'
NcoI XbaI

RevGiII: 5' GC ***GAA TTC*** TTA AAA GAG ACC ACA ATC 3'
EcoRI

PCRCFP:

CFPFwd: 5' CAT A***CC ATG G***TG AGC AAG GGC GAG GAG 3'
NcoI

CFPRev: 5' TTC ***TCT AGA*** CTT GTA CAG CTC GTC CAT 3'

A.9 Cloning region of pVLMelFlagHis10TevRecStrepII

Honey bee Melittin signal ↓

…..ATG AAA TTC TTA GTC AAC GTT GCC CTT GTT TTT ATG GTC GTG TAC ATT TCT TAC ATC ACT

Flag Tag

GCA GAC TAC AAG GAC GAT GAC GAT AAG GAT

His Tag TEV Cleavage site

CAT CAC CAT CAC CAT CAC CAT CAC CAT CAC GG *G GAT CG* A ATC CCT ACA ACT GAA AAC CTG TAC TTT CAA GGT

Dead BamHI

Strep Tag II

CAG GAT CCG CTnnnnnnnnnnnn GAA TTC TGG AGT CAT CCT CAA T TT GAA AAA TAA GCG GCC GC…….

BamHI EcoRI NotI

Bio-domain Seq

GAA TTC GCG GCG GCA CCG GCG GCG CGC CGG CAC CGG CAG CAG GTG GCG CAG GCG CCG GTA AGG CCG GAG

EcoRI

AGG GCG AGA TTC CCG CTC CGC TGG CCG GCA CCG TCT CCA AGA TCC TCG GCG GCC GC

NotI

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Résumé

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Publications:

1. **Kapoor M, Chandramouli Reddy C, Krishnasastry MV, Surolia N and Surolia A.** Slow-tight-binding inhibition of enoyl-acyl carrier protein reductase from *Plasmodium falciparum* by triclosan. ***Biochem J.*** *2004 August1; 381:719-724*

2. **Chandramouli Reddy C, Reinhart C and Michel H.** C-terminal truncated cannabinoid receptor 1 and the G protein trimer co-expressed heterologously in *Sf9* cells are precoupled and show constitutive activity. *Manuscript in preparation.*