



Construction of a recombinant mu opioid receptor tagged with FLAG epitope and yellow fluorescent protein to generate a FLIP-In HEK293 stable cell line

Author: Nuseibah Hasan Flayyeh AL Qtaish.

Scientific Supervisor: Dr. Juan Francisco López-Giménez.

Institute of Biomedicine and Biotechnology of Cantabria (IBBTEC).

Molecular Biology and Biomedicine Master Program 2014/2015. University of Cantabria, Santander, Spain.

Introduction

G-protein-coupled receptors

G protein coupled receptors (GPCRs) represent the single largest class of membrane proteins in the human genome [1]. A recent and detailed analysis of the human genome reveals over 800 unique GPCRs, of which approximately 460 are predicted to be olfactory receptors. Based on sequence similarity within the 7 TM segments, these receptors can be clustered into 5 families: the rhodopsin family (701 members), the adhesion family (24 members), the frizzled/taste family (24 members), the glutamate family (15 members), and the secretin family (15 members). The physiologic function of a large fraction of these 800 GPCRs is unknown; these receptors are referred to as orphan GPCRs. They are a promising group of targets for the pharmaceutical industry.

GPCRs share a common structural signature of seven hydrophobic transmembrane (TM) segments, with an extracellular amino terminus and an intracellular carboxyl terminus. GPCRs share the greatest homology within the TM segments. The most variable structures among the family of GPCRs are the carboxyl terminus, the intracellular loop spanning TM5 and TM6, and the amino terminus. The greatest diversity is observed in the amino terminus. This sequence is relatively short (10–50 amino acids) for monoamine and peptide receptors, and much larger (350–600 amino acids) for glycoprotein hormone receptors, and the glutamate family receptors. The largest amino terminal domains are observed in the adhesion family receptors.

GPCRs mediate senses such as odor, taste, vision, and pain in mammals [2]. In addition, important cell recognition and communication processes often involve GPCRs. Indeed, many diseases involve malfunction of these receptors, making them important targets for drug development. Unfortunately, despite their importance there is insufficient structural information on GPCRs for structure-based drug design. This is because these membrane-bound proteins are difficult to crystallize, and the atomic-level structure has been solved only for bovine rhodopsin. Consequently, it is important to develop theoretical methods to predict the structure and function of GPCRs. Experimental data relevant to the function of GPCRs is available for ligand activation of GPCRs and site-directed mutagenesis. This data has led to information about structural features in the ligand-binding regions of GPCRs. Protein sequence analyses on GPCRs reveals a common protein topology consisting of a membrane-spanning seven-helix bundle, which likely accommodates the binding site for low-molecular-weight ligands. Structurally, GPCRs can be classified as (i) GPCRs with short N terminus (5–80 residues) and (ii) GPCRs with a long N-terminal ectodomain (80–600 residues). The long N terminus of class II GPCRs may be involved in the ligand recognition, but ultimately the bound ligand probably moves into the transmembrane (TM) region to activate the G protein.

The biological and medical importance of GPCRs is well established and extensively documented [3]. The breadth of GPCR distribution across nearly all of the body's organs and tissues and the cellular role

GPCRs play as signal transducers make GPCRs key regulatory elements in a broad range of normal and pathological processes. Thus, GPCRs have been and will continue to be an important focus for drug discovery.

The medicinal importance of GPCRs can be partially appreciated by considering their location and function within the cell [3]. The physical location and disposition of GPCRs spanning the cell's plasma membrane connect extracellular and intracellular environments, providing a direct mechanism for the transduction of extracellular messages into intracellular responses. In this way and together with their transmitters and effectors, GPCR systems function to modulate a broad spectrum of cellular phenomena dictated by the needs of the tissues and organs they serve. Common biological actions attributed to GPCRs include but are not limited to the following: modulation of neuronal firing, regulation of ion transport across the plasma membrane and within intracellular organelles, modulation of homeostasis, control of cell division/proliferation, and modification of cell morphology.

Over the past decade, the pursuit of GPCRs as targets for drug discovery campaigns has benefited greatly from the development and adoption of high-throughput approaches to their pharmacological assay and medicinal chemistry. Availability of these tools in conjunction with a genomically complete GPCR target palette has effectively enabled researchers to rapidly screen GPCRs of specific therapeutic interest and quickly elaborate upon potential leads during the ensuing drug development process, thus sparking a renaissance in GPCR pharmacology.

In terms of structure, GPCRs are characterized by an extracellular N-terminus, followed by seven transmembrane (7-TM) α -helices (TM-1 to TM-7) connected by three intracellular (IL-1 to IL-3) and three extracellular loops (EL-1 to EL-3), and finally an intracellular C-terminus. The GPCR arranges itself into a tertiary structure resembling a barrel, with the seven transmembrane helices forming a cavity within the plasma membrane that serves a ligand-binding domain that is often covered by EL-2. Ligands may also bind elsewhere, however, as is the case for bulkier ligands (e.g., proteins or large peptides. The eventual effect of of agonist-induced activation is a change in the relative orientations of the TM helices (likened to a twisting motion) leading to a wider intracellular surface and revelation of residues of the intracellular helices and TM domains crucial to signal transduction function (i.e., G-protein coupling). Inverse agonists and antagonists may also bind to a number of different sites, but the eventual effect must be prevention of this TM helix reorientation [4].

Although the details of GPCR signaling in aggregate are complex, the basic tenets that describe the initial interaction of the receptor with its proximal partner, the G protein heterotrimeric complex, are straightforward. Upon adoption of an "active" conformation (most simply envisioned as the result of agonist binding), the intracellular domains of a GPCR interact with a membrane-associated GDP-charged G protein heterotrimeric complex (G $\alpha\beta\gamma$).This heterotrimeric complex then undergoes GTP/GDP exchange with subsequent dissociation of G α and G $\beta\gamma$ subunits that in turn interact with specific downstream intracellular effector systems. Activation of multiple heterocomplexes as well as

 $G\alpha$ cycling through active and inactive configurations via a GTP hydrolysis cycle provides immediate amplification and temporal regulation of the initial receptor-ligand signaling event. In due course, through the process of desensitization, the active conformation of the receptor is blocked and signaling is attenuated by agonist dissociation and/or deactivation through interaction with β -arrestins in response to activation-specific phosphorylation by G protein-coupled receptor kinases and/or internalization. The immediate activities of these effector systems fall into four main categories: stimulation of cAMP production, inhibition of cAMP production, stimulation of phospholipase C with subsequent mobilization of intracellular Ca²⁺, and activation of plasma membrane proton flux. These phenomena are controlled by which class of G α subunit is activated. There are at least 16 human G α subunits, 5 G β subunits, and 11 G γ subunits. In addition to G α -controlled events, the G $\beta\gamma$ subunits also can regulate their own effectors, including additional forms of adenylate cyclase as well as ion channels. The ramifications of signaling complexity implicit in the full range of combinatorial permutations within the heterotrimeric complex itself have yet to be fully examined [3].

The desensitization of a G protein-coupled receptor (GPCR) response can be described as the loss of response subsequent to prolonged or repeated administration of an agonist [5]. Actually the term 'prolonged' can be somewhat misleading as experimentally this can represent time periods of as little as a few seconds or as long as several hours or even days. Desensitization can be homologous or heterologous in nature; homologous desensitization refers to the loss of response solely to agonists that act at a particular GPCR subtype, whereas heterologous desensitization refers to a more generalized effect involving the simultaneous loss of agonist responsiveness at multiple GPCR subtypes even in the absence of agonist occupation of the other receptors. Homologous desensitization is usually thought to involve adaptive changes at the level of the GPCR itself, whereas heterologous desensitization may also involve changes in signalling components downstream of the GPCR. Following desensitization, and provided that agonist stimulation is curtailed by removal of agonist or addition of an antagonist, GPCR responsiveness can in most cases be regained by a process called resensitization, although as with desensitization, the rapidity of this process varies between GPCR subtypes and can also depend upon the length of agonist pretreatment in the desensitization phase. Furthermore, in pharmacology, desensitization has a different meaning from downregulation, the latter referring to the proteolytic degradation of GPCRs, often in lysosomes. Thus, although downregulation of a GPCR invariably adds to the overall desensitization of a GPCR response, most GPCRs can undergo extensive desensitization (particularly, following acute agonist addition) without any downregulation being detectable.

The increased evidence of compartmentalisation of GPCR mediated signalling as a consequence of scaffolding in larger molecular complexes raises the prospect of microdomain-specific signalling and pharmacology [6]. Our increased knowledge of receptor location must also be matched to the accumulating evidence for agonist specific signalling and the importance of both temporal and spatial aspects of intracellular signalling. As a consequence, there is the real prospect that the molecular

pharmacology of a given ligand-receptor interaction may differ between microdomains within a single cell. The challenge for the future is to develop the technology to be able to study receptors in these domains. Fluorescent ligands and GFP-tagged GPCRs have been developed for this purpose and recent advances include our own application of fluorescence correlation spectroscopic techniques to real-time measurement of ligand binding to GPCRs in membrane microdomains and the FRET-based approaches developed by Martin Lohse to monitor real-time changes in receptor conformation. Classical pharmacology has in the past provided the essential means by which quantitative studies of ligand affinity and efficacy can be measured using indirect means. The challenge for the future will be to apply and adapt the same rigorous approaches, in collaboration with medicinal chemists and molecular biologists, to interrogate ligand-receptor interactions at the single molecular level in real time and in living cells.

Mu opioid receptors:

Opioid receptors have been targeted for the treatment of pain and related disorders for thousands of years, and remain the most widely used analgesics in the clinic. Mu (μ), kappa (κ), and delta (δ) opioid receptors represent the originally classified receptor subtypes, with opioid receptor like-1 (ORL1) being the least characterized. All four receptors are G-protein coupled, and activate inhibitory G-proteins. These receptors form homo- and hetereodimeric complexes, signal to kinase cascades, and scaffold a variety of proteins [7].

Opioids are the most widely used and effective analgesics for the treatment of pain and related disorders. Opiates have been used for thousands of years for the treatment of pain, and in the last century we have made huge strides in the development of opioids derived from naturally occurring opiates within the fields of receptor pharmacology and medicinal chemistry. In addition to pain, opioids are frequently used in the treatment of numerous other disorders including diarrhea, cough, post-operative pain and cancer.

Opioid systems are critical in the modulation of pain behavior and antinociception. Opioid peptides and their receptors are expressed throughout the nociceptive neural circuitry in addition to critical regions of the central nervous system included in reward and emotion-related brain structures. The most commonly used opioids for pain management act on μ opioid receptor (MOP) systems. While μ opioids continue to be some of the most effective analgesics, they are also efficacious mood enhancers and cause activation of central dopamine reward pathways that modulate euphoria. These unwanted side effects have driven researchers at basic and clinical levels to actively pursue other opioid receptors as putative drug targets for pain relief.

The mu opioid receptors (MOP) are a class of opioid receptors. Named after morphine, the mu opioid receptor is the physiological target of such potent analgesics as morphine and fentanyl, as well as the

endogenous opioid peptides, β -endorphin, enkephalins, and dynorphins. Opioid drugs with high abuse liability such as morphine, methadone, and fentanyl all bind the mu receptor with high affinity.

The MOP is known to exert two types of inhibitory effects on a cell reduction of the intracellular level of cyclic adenosine monophosphate (cAMP) and inhibition of neuronal firing. Using the cloned mu opioid receptors, the authors have begun to study the molecular mechanisms for both of these processes. The mu opioid receptors contain seven transmembrane hydrophobic domains, a structural motif most commonly found in G-protein-coupled receptors. This suggests that the mu opioid receptors may couple to heterotrimeric G-proteins to mediate intracellular signal transduction. Previous studies using cell lines that constitutively express endogenous opioid receptors also suggest that opioid receptors are 109 coupled to G-proteins. Upon activation of a G-protein, the alpha subunit of the Gprotein dissociates and displays an increase in its intrinsic GTPase activity. To test whether the cloned mu opioid receptor couples to G-proteins, GTPase activities from cell membranes expressing the mu receptor were measured in the presence and absence of mu-selective ligands. Treatment of CHO cells stably expressing the mu receptor with the unselective agonist DAMGO elevated the GTPase activity by 33percent. This stimulation was blocked by the opioid antagonist naloxone. In nontransfected parental CHO cells, on the other hand, GTPase activity was not affected by DAMGO treatment. Because an increase in the low affinity GTPase activity is indicative of G-protein activation, these results suggest that the mu opioid receptor is functionally coupled to the G proteins in these mammalian cells.

MOPs are G protein-coupled receptors signaling primarily to inhibit adenylyl cyclase and regulate the opening of K^+ and Ca^{2+} ion channels. After activation of MOP-r by full agonists such as the enkephalin analog DAMGO or the synthetic drug sufentanil, the receptor is phosphorylated and then internalized. These processes are linked but can be separated. Internalization of the receptor removes a significant fraction of functional MOP-r from the plasma membrane and thus may contribute in part to the loss of agonist signaling (tolerance) induced by high or sustained agonist exposures. Receptor phosphorylation after exposure to morphine and some other clinically useful analgesics occurs at a much slower rate than after exposure to full agonists, and morphine induces little internalization of the receptor. Once internalized after full agonist exposure, a region of the MOP-r sequence directs the internalized receptors to a re-cycling pathway that ultimately returns de-phosphorylated receptors back to the plasma membrane with restored functionality, where they are immediately available to re-initiate agonist signaling. It has been unclear whether this recycling process is rapid enough to permit recovery of signaling during a single exposure to high concentrations of full agonist. An article in the current issue of Molecular Pharmacology sheds further light on the role of agonist-induced internalization of MOP-r in maintaining agonist signaling.

Opiates are among the most effective analgesics known but their clinical use is limited by severe side effects [8]. Some of these undesired actions including tolerance, dependence and abuse usually appear after repeated opioid administration, and have been linked to adaptations that take place in order to

counteract prolonged opioid receptor activation. Adaptive changes have been described at different organizational levels within the central nervous system, ranging from receptor and cellular alterations to functional modifications of different neuronal networks. Regulation that occurs at the receptor level results in the progressive waning of signalling efficacy and is known as desensitization. Mechanisms of opioid receptor desensitization were initially characterized in immortalized cell lines but more recent studies have extended observations to cultured neurons and animal models.

Studies in immortalized cell lines have shown that like for many other GPCRs, opioid receptor activation involves a series of conformational changes that trigger signalling and regulation. Regulatory steps usually start with phosphorylation of the receptor followed by βarrestin recruitment and disruption of receptor signaling via G-protein coupled effectors. In addition, since arrestins bind to the coat structure of clathrin-coated pits a great majority of ligands that promote functional desensitization also enhance sequestration. The frequent association of these two processes was initially taken as an indication that opioid receptor internalization and desensitization were causally linked, an interpretation that was reinforced by studies showing that morphine failed to induce both, internalization and desensitization. Moreover, given that morphine induces more analgesic tolerance than agonists capable of triggering a full regulatory response, its high potential for tolerance was initially considered as the consequence of cellular adaptations to counteract sustained signaling by receptors that were unable to desensitize or internalize.

To explore potential cross-regulation between these G protein-coupled receptors, the human μ opioid peptide receptor was expressed stably and constitutively in Flp-In T-REx human embryonic kidney 293 cells that harbored the human 5-HT_{2A} receptor at the inducible Flp-In locus [9]. In the absence of the 5-HT_{2A} receptor, pretreatment with the enkephalin agonist [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin but not with the alkaloid agonist morphine produced desensitization, internalization, and down-regulation of the µ opioid peptide receptor. Induction of 5-HT_{2A} receptor expression in these cells resulted in upregulation of μ opioid peptide receptor levels that was blocked by both a 5-HT_{2A} receptor inverse agonist and selective inhibition of signaling via $G\alpha_{d}/G\alpha_{11}$ G proteins. After induction of the 5-HT_{2A} receptor, coaddition of 5-HT with morphine now also resulted in desensitization, receptor internalization, and down-regulation of the μ opioid peptide receptor. It has been argued that enhancement of μ opioid peptide receptor internalization in response to morphine would limit the development of tolerance without limiting analgesia. Morphine is used widely as an analgesic in the treatment of chronic pain. However, tolerance to morphine develops rapidly, restricting its clinical utility. The analgesic effects of morphine are clearly mediated via the μ opioid peptide (MOP) receptor because they are absent in animals lacking this GPCR. However, despite a vast range of studies that have attempted to understand the molecular basis of tolerance to morphine and that have explored why other agonists that also activate the MOP receptor have different functional profiles, this remains a contentious area generating many, apparently conflicting, views. It was assumed initially that the development of tolerance to morphine would reflect MOP receptor desensitization, which would be anticipated to preclude sustained function. Indeed, recent studies have suggested that repeated morphine administration can enhance agonist potency and hence increase receptor desensitization and promote tolerance. In contrast, a series of studies have suggested that tolerance to morphine may stem from a lack of rapid desensitization, resulting in other adaptive and potentially slowly reversible, and changes becoming dominant.

Heterologous Expression Systems

The expression of cloned genes in mammalian cells is a basic tool for understanding gene expression, protein structure, and function, and biological regulatory mechanisms [10]. The level of protein expression from heterologous genes introduced into mammalian cells depends upon multiple factors including DNA copy number, efficiency of transportation, mRNA processing, mRNA transport, mRNA stability, and translational efficiency, and protein processing, transport, and stability. Different genes exhibit different rate limiting steps for efficient expression. Multiple strategies are available to obtain high level expression in mammalian cells.

Gene is defined as a discrete unit of genetic information which is required for the production of a polypeptide. It includes the coding sequence, the promoter and terminator, and introns. Expression can be described as a transcription and translation of a gene [11]. A particular host is required to express a particular gene. Today, there are wide selections of expression systems available for large-scale recombinant protein production. These expression systems include E. coli, baculovirus-mediated insect cell expression, yeast, and several mammalian based systems. Each has its own respective advantages in relation to cost, ease of use, and their post-translational modification profiles.

A number of mammalian cell lines have been utilized for protein expression with the most common being HEK 293 (Human embryonic kidney) and CHO (Chinese hamster ovary). These cell lines can be transfected using polyethyleneimine (PEI) or calcium phosphate. HEK 293 cells exhibit the highest level of PEI-mediated transfection with 50–80% of cells showing green fluorescent protein (GFP) expression, and are now widely used for production of recombinant proteins both by transient transfection as well as by the formation of stable cell lines.

Protein expression in mammalian cells can also be achieved using viral-mediated transduction by such techniques as the BacMam system. This technology utilizes recombinant baculoviruses for simple transduction of mammalian cells, allowing for production of milligram quantities of protein for structural studies. Other cell lines such as COS and Vero (both green African monkey kidney), HeLa (Human cervical cancer), and NS0 (Mouse myeloma) have also been used for structural studies. Some of these cell lines such as NS0 are more difficult to transfect. Transfection can be usually achieved using electroporation, and are only used in stable cell line production. The main advantages of mammalian cell expression are that the signals for synthesis, processing and secretion of eukaryotic proteins are properly and efficiently recognized by the mammalian cells. However, it should be noted that there are differences between species.

The development of recombinant cDNA methodology, combined with a wide range of molecular and cell biology techniques, has made feasible the expression of particular genes of interest into a vast variety of host cells. Heterologous expression systems provide a cellular context in which properties of a given receptor may be studied in the absence of the native intricate background, alleviating the complexity due to the presence of multiple receptor sub-types and/or to coupling to more than one class of G- proteins. In most cases, heterologous expression allows the analysis of single receptor subtypes in a defined system, and in addition provides easy access to human receptors outside their native tissues. Particularly in our field of interest, such heterologous expression systems have been extremely useful to investigate pharmacological and molecular properties of each opioid receptor subtype when expressed individually. Furthermore, these experimental models allow the expression of genetically modified receptors in a way that facilitates their study, for example containing a short amino acid sequence as an epitope that is recognized specifically by an antibody. The use of such epitope-tagged receptors has provided means to employ biochemical techniques to facilitate the cellular and molecular studies of these GPCRs.

Fluorescent proteins

Fluorescent Proteins are members of a structurally homologous class of proteins that share the unique property of being self-sufficient to form a visible wavelength chromophore from a sequence of 3 amino acids within their own polypeptide sequence. It is common research practice for biologists to introduce a gene (or a gene chimera) encoding an engineered fluorescent protein into living cells and subsequently visualize the location and dynamics of the gene product using fluorescence microscopy. The discovery and development of fluorescent proteins from a wide variety of organisms has initiated a revolution in the study of cell biology by providing convenient markers for gene expression and protein targeting in living cells and organisms. The resulting fusion product can be used to localize previously uncharacterized proteins or to visualize and track known proteins to further understand cellular events. The use of fluorescent proteins as a minimally invasive tool for studying protein dynamics and function has been stimulated by the engineering of genetic variants with improved brightness, photostability and expression properties. Cells that express gene products tagged with fluorescent proteins can be imaged with low light intensities over many hours to provide useful information about changes in the steady-state distribution of a protein over time.

The field of fluorescent protein research has progressed from squeezing jellyfish through cloth bags to the creation of transgenic mice with rainbow brains. During the course of this progression, the fluorescent proteins have been studied, modified, and applied to an extent that is enjoyed by only a handful of other classes of proteins. This attention is clearly warranted due to the incalculable value of fluorescent proteins for the study of cells, tissues, and even whole animals at level of detail and subtlety that would otherwise be experimentally inaccessible. Unfortunately, *Aequorea victoria* has not enjoyed a similar level of interest, and in recent years there has been scant research on this animal that gave science so much. Fluorescent proteins are genetically encoded, easily imaged reporters crucial in

biology and biotechnology. When a protein is tagged by fusion to a fluorescent protein, interactions between fluorescent proteins can undesirably disturb targeting or function. Unfortunately, all wild-type yellow-to-red fluorescent proteins reported so far are obligately tetrameric and often toxic or disruptive.

Yellow fluorescent protein (YFP) is a genetic mutant of green fluorescent protein (GFP) originally derived from the jellyfish Aequorea victoria. Its excitation peak is 514 nm and its emission peak is 527 nm. Like the parent GFP, YFP is a useful tool in cell and molecular biology thanks to its properties useful for fluorescence microscopy [12].

Three improved versions of YFP are Citrine, Venus, and Ypet. They have reduced chloride sensitivity, faster maturation, and increased brightness (defined as the product of the extinction coefficient and quantum yield). Typically, YFP serves as the acceptor for genetically-encoded FRET sensors of which the most likely donor FP is monomeric cyan fluorescent protein (mCFP). The red-shift relative to GFP is caused by a Pi-Pi stacking interaction as a result of the T203Y substitution introduced by mutation, which essentially increases the polarizability of the local chromophore environment as well as providing additional electron density into the chromophore.

The green fluorescent protein (GFP) from the jellyfish Aequorea victoria has provided a myriad of applications for biological systems [13]. Over the last several years, mutagenesis studies have improved folding properties of GFP. However, slow maturation is still a big obstacle to the use of GFP variants for visualization. These problems are exacerbated when GFP variants are expressed at 37°C and/or targeted to certain organelles. Thus, obtaining GFP variants that mature more efficiently is crucial for the development of expanded research applications. Among Aequorea GFP variants, yellow fluorescent proteins (YFPs) are relatively acid-sensitive, and uniquely quenched by chloride ion (Cl-). For YFP to be fully and stably fluorescent, mutations that decrease the sensitivity to both pH and Cl- are desired. Here we describe the development of an improved version of YFP named "Venus". Venus contains a novel mutation, F46L, which at 37°C greatly accelerates oxidation of the chromophore, the ratelimiting step of maturation. As a result of other mutations, F64L/M153T/V163A/S175G, Venus folds well and is relatively tolerant of exposure to acidosis and Cl-. We succeeded in efficiently targeting a neuropeptide Y-Venus fusion protein to the dense-core granules of PC12 cells. Its secretion was readily monitored by measuring release of fluorescence into the medium. The use of Venus as an acceptor allowed early detection of reliable signals of fluorescence resonance energy transfer (FRET) for Ca2+ measurements in brain slices. With the improved speed and efficiency of maturation and the increased resistance to environment. Venus will enable fluorescent labelings that were not possible before.

The production of proteins in appropriate quantity and quality is an essential requirement of the present time. There appears to be a progressive increase in the application of mammalian cells for proteins production. Expression systems utilizing mammalian cells for recombinant proteins are able to introduce proper protein folding, post-translational modifications, and product assembly, which are important for complete biological activity.

Mutation appearance in the DNA sequence

A mutation, which may arise during replication and/or recombination, is a permanent change in the nucleotide sequence of DNA [14]. Damaged DNA can be mutated either by substitution, deletion or insertion of base pairs.

Mutations might be caused by ;(1) Error in DNA Replication, on very, very rare occasions DNA polymerase will incorporate a noncomplementary base into the daughter strand. During the next round of replication the missincorporated base would lead to a mutation. This, however, is very rare as the exonuclease functions as a proofreading mechanism recognizing mismatched base pairs and excising them, (2) Errors in DNA Recombination DNA often rearranges itself by a process called recombination which proceeds via a variety of mechanisms. Occasionally DNA is lost during replication leading to a mutation, (3) Chemical Damage to DNA, many chemical mutagens, some exogenous, some man-made, some environmental, are capable of damaging DNA. Many chemotherapeutic drugs and intercalating agent drugs function by damaging DNA, (4) Radiation, gamma rays, X-rays, even UV light can interact with compounds in the cell generating free radicals which cause chemical damage to DNA.

Several types of mutations are known: (1) the substitution of one base pair for another, (2) the deletion of one or more base pairs, and (3) the insertion of one or more base pairs. The spontaneous mutation rate of T4 phage is about 10-7 per base per replication. E. coli and Drosophila melanogaster have much lower mutation rates, of the order of 10-10. The substitution of one base pair for another is a common type of mutation.

Two types of substitutions are possible. A transition is the replacement of one purine by the other or that of one pyrimidine by the other. In contrast, a transversion is the replacement of a purine by a pyrimidine or that of a pyrimidine by a purine. A single base substitution is the most common type of mutation and there are two types; transition: this occurs when a purine is substituted with another purine or when a pyrimidine is substituted with another pyrimidine and transversion, when a purine is substituted for a pyrimidine or a pyrimidine replaces a purine.

Point mutations that occur in DNA sequences encoding proteins are either silent, missense or nonsense. Silent, If abase substitution occurs in the third position of the codon there is a good chance that a synonymous codon will be generated. Thus the amino acid sequence encoded by the gene is not changed and the mutation is said to be silent, and missence when base substitution results in the generation of a codon that specifies a different amino acid and hence leads to a different polypeptide sequence. Depending on the type of amino acid substitution the missense mutation is either conservative or nonconservative. For example if the structure and properties of the substituted amino acid are very similar to the original amino acid the mutation is said to be conservative and will most likely have little effect on the resultant proteins structure. If the substitution leads to an amino acid with very different structure and properties the mutation is nonconservative and will probably be deleterious

(bad) for the resultant proteins structure, and nonsense, when a base substitution results in a stop codon ultimately truncating translation and most likely leading to a nonfunctional protein.

Objectives

Opioid receptors are widely involved in various physiological and pathophysiological activities, opioid receptors play an essential role in the different cellular signaling pathways and physiological effects, therefore we have the main purpose of our research is construction of a recombinant mu opioid receptor tagged with FLAG epitope and yellow fluorescent protein to generate a future FLIP-In HEK 293 stable cell line and to provide MOP sample for better understanding the mu opioid function and better studying the gene expression. Many tasks required to achieve our objectives.

- Construction of a recombinant mu opioid receptor tagged with FLAG epitope and yellow fluorescent protein to be expressed in human embryonic kidney 293 cells.
- Mutation detection and repair in the DNA sequence of mu opioid receptor.
- Transient expression of mu opioid receptor in HEK 293 cells.
- Performing various molecular biology techniques.
- Application of fluorescence microscopy studies.

Methods

Using molecular biology techniques in our investigation is divided into four parts. The first part is devoted to techniques for Subcloning of FLAGMOPYFP into pcDNA5, from the isolation of the DNA to the sequencing of entire comparative genomics. The second part is concerned with the mutation detection and repair in the sequence of our construct. The third part is concerned with the cell culture to generate a transient expression of MOP in HEK293 cell line. The final part is concerned with the application of fluorescence microscopy studies.

Subcloning of FLAGMOPYFP insert into pcDNA5

Subcloning of FLAGMOPYFP into pcDNA5 was a basic step in our project is required to move the FLAGMOPYFP insert from pcDNA3 vector to pcDNA5 vector to gain the desired functionality to study our insert. Essentially all subcloning reactions proceed the same way as illustrated in figure (1).



Figure (1): Subcloning of FLAGMOPYFP insert from the parent vector pcDNA3 into pcDNA5 vector.

We have released and purified our insert from the parent vector pcDNA3 by digestion of FLAGMOPYFPpcDNA3 with Hindlll and Xho l (see figure 2). In parallel, the vector pcDNA5 was also digested with Hindlll and Xhol, and subsequently the insert FLAGMOPYFP and the vector pcDNA5 were ligated. The ligation reaction was transformed into competent bacterial cells. Then the transformant cells underwent miniprep and maxiprep techniques to isolate the cDNA at the purity and scale we need.



Figure (2): FLAGMOPYFPpcDNA3 was the starting sample for our investigation. To purify FLAGMOPYFP insert from pcDNA3 vector; we have to digest the construct with hindll and xhol enzymes.

The first task was isolation the insert FLAGMPOYFP to have the desired insert, to isolate the insert from the entire construct we had to digest the construct, the process of cutting DNA molecules into smaller pieces with special enzymes called Restriction Enzymes. Digestion begin by mixing the DNA and the Restriction Enzymes, The actual reaction conditions vary from one enzyme to the next, many of the variables are optimized by mixing the enzyme and DNA with a buffer specific for the enzyme of choice. The restriction enzymes that can cut the insert FLAGMOPYFP from the construct FLAGMOPYFP pcDNA3 are Hindlll and Xhol and the specific buffer for these enzymes is buffer B.

Once the digestion was completed, agarose gel electrophoresis was performed to separate the digested fragments by size and visualize the fragments and purify them for further experiments. After that DNA samples were run on a preparative agarose gel to extract the desired DNA samples, extraction involves four basic steps: identifying the fragments of interest, isolating the corresponding bands, isolating the DNA from those bands, and removing the accompanying salts and stain.

After extraction, fragments of interest can be mixed, precipitated, and enzymatically ligated together in several simple steps. Ligation is an essential laboratory procedure in the molecular cloning of DNA whereby DNA fragments are joined together to create recombinant DNA molecules. The ends of DNA fragments are joined together by the formation of phosphodiester bonds between the 3'-hydroxyl of one DNA terminus with the 5'-phosphoryl of another, is normally performed by using T4 DNA ligase, then we transformed and plated the ligation reaction into competent bacteria, Bacteria are commonly used as host cells for making copies of DNA in the lab because they are easy to grow in large numbers. Their cellular naturally carries out DNA replication and protein synthesis. This gives them an evolutionary advantage and helps them survive changes in their environment. For example, bacteria can acquire DNA that makes them resistant to antibiotics. The purpose of this technique is to introduce a foreign

plasmid into competent bacteria and to use those bacteria to amplify the plasmid in order to make large quantities.

We performed plasmid miniprep and maxiprep preparation to isolate plasmid DNA at the purity and scale we need, miniprep usually is performed for few ml of the DNA sample, to get around 10 μ g of DNA plasmid and maxiprep is performed for 100 ml, we get around 500 μ g of DNA plasmid. After performing miniprep and maxiprep the concentration of our sample was = 2.33 μ g/ μ l.

We prepared the sample with the proper primers that cover all the sequence of FLAGMOPYFPpcDNA5:

1. Primer MOPseq1fw (5 ' ATG GAC AGC AGC GCT GCC CCC 3 ') annealing forward at the first 1000 bases.

2. Primer MOPseq1000fw (5 ' AAC CCA GTC CTT TAT GCA TTT 3') annealing forward from base number 1000 to base number 1970.

3. Primer MOPseq1360rev (5 ' GAG CTC TCT GGC TAA CTA GAG 3') annealing reversely from base number 1360 to base number 790.

4. Primer MOPseq1970rev (5 ' GGC TGG CAA CTA GAA GGC ACA 3') annealing reversely from base number 1970 to base number 1050.

Then we sent the sample with each primer for sequencing reactions service (<u>http://www.stabvida.com</u>) to make sure if the prepared sample had the correct expected construct, we analyzed the result of sequencing using PubMed Nucleotide BLAST alignment sequences and serial cloner program.

Problem

The sequencing reactions resulted in a mutation in our construct at position 210 (see figure 3), the original proline (CCT) was substituted by leucine (CTT).



Figure (3): After subcloning of FLAG insert into pcDNA5 vector, we found that our construct has a mutation at position 210.

Analysis of the available crystal structures of class A GPCRs has shown that highly conserved hydrophobic amino acids also play an important role in receptor activation, G protein coupling, and

receptor oligomerization, indicating that most of these amino acids are involved in maintaining the three dimensional structure of the receptor and in the processes of receptor activation. Both of Leucine and proline amino acids are hydrophobic (see table.1).

Properties	Leucine	Proline	
Molecular formula	C6H13NO2	C5H9NO2	
Acidity (pKa)	2.36 (carboxyl) 9.60 (amino)	1.99 (carboxyl) 10.96 (amino)	
Molar mass	131.17 g·mol-1	115.13 g·mol−1	
Structure	H ₂ N OH	ОН	

Table (1): Studying the differences between Leucine and Proline amino acids.

Site-directed mutagenesis method to correct leucine mutation:

A close physical relationship between extracellular loop (EL) and the ligand binding pocket of MOP explains why mutating specific residues in EL can alter subtype selectivity of ligands(see figure 4).



Figure (4): Using protein data bank and uniprot web pages, and pymol program, we have examined the detailed structure of our construct; we found that the amino acid at position 210 is located in the extracellular region.

We performed site-directed mutagenesis method to correct leucine mutation at position 210 in our sequence. By using two synthetic oligonucleotide primers containing the desired change, i.e. substitution of CTT by CTT, we made PCR reaction under a specific program for FLAGMOPYFP L210P that contains the undesired mutation with the designed primers that contain the desired change. The PCR reaction product was digested with DpnI to cut the methylated, parental DNA template, and then we transformed the digested product into competent cells to get the correct construct of FLAGMOPYFP (see Figure 5)



Figure (5): Site-directed mutagenesis method to correct the mutation.

Site-directed mutagenesis method performed using PfuTurbo DNA polymerase and a temperature cycler. PfuTurbo DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired change.

We designed two primers containing the correct base sequence (33 base long), both primers should contain the desired mutation and anneal to the same sequence on opposite strands, the primers should be between 25 and 45 bases in length, with melting temperature equal or more than 78 C, the desired change should be in the middle of the primer.

```
Primer MOP630Cfw: 5 'TCA GCC ATT GGT CTT CCT GTA ATG TTC ATG GCT 3'
```

Primer MOP630Crev: 5 'AGC CAT GAA CAT TAC AGG AAG ACC AAT GGC TGA 3 '

Then we made PCR reaction under a specific program (see table 2), PCR reaction was applied to our construct FLAGMOPYFP L210P that contain the undesired mutation, the new designed primers that contain the desired change and pfu DNA polymerase the enzyme that serves the central function of copying a new strand of DNA during each extension step. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by Pfu DNA polymerase. Incorporation of the oligonucleotide primers generates a desired mutated plasmid containing staggered nicks.

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12–18	95°C	30 seconds
		55°C	1 minute
		68°C	1 minute/kb of plasmid length*

Table (2): Cycling parameters for the site-directed mutagenesis method.Cycle the reaction using the cycling parameters outlined, segment 2 of the cycling parameters should be adjusted in accordance with the type of mutation desired, for our construct which has single amino acid change, should be 16 cycles.

After that the PCR reaction was cleaned up and the DNA was eluted with distilled water. Following the reaction, the product was digested with DpnI, this digest is crucial; DpnI only cleaves at methylated sites, so it chews up the parental template DNA plasmid but not the PCR product. Since the transformation efficiency of the circular template plasmid is several orders of magnitude better than the linear PCR product, without the DpnI digest a large number of the colonies would be the parental, this means that the template plasmid cannot come from a methylation deficient strain.

A high fidelity polymerase is also crucial; the PCR reaction goes around the entire plasmid, so we need to minimize the chances of introducing unwanted mutations in both our gene and the backbone. A "hot start" formulation of the enzyme is desirable, as the proof-reading capability of most of these enzymes may otherwise degrade the primers during setup.

We transformed the digested product into competent cells, 4 colonies were Picked up in 4 tubes with LB and ampicillin and incubated at 37 C overnight, the samples underwent miniprep technique for each sample, we made digestion reaction for each sample, run the forth digested samples in the gel and we get the image to decide which one of the samples is better to perform maxiprep technique (FLAGMOPYFPpcDNA5 concentration = $1.7 \mu g/\mu l$), then we ordered two primers to sequence the sample to check for our mutation and any PCR introduced errors. The sequencing resulted that the mutation was corrected.

Transient transfection of mu opioid receptor in HEK 293 cells

Mammalian cells are used as a host for gene expression in order to confirm that cloned genes can direct the synthesis of desired proteins, to study protein structure-function relationships, to isolate genes by direct screening or selecting transfected cells that express a desired protein, to produce proteins that are available in limited quantity; and to evaluate the physiologic consequences of expression of specific proteins in mammalian cells in order to study biological regulatory control mechanisms [10].

The technology of expressing foreign genes in mammalian cells has become increasingly important to study a number of biological questions and as a primary method for production of proteins for pharmaceutical use. Mammalian cells are frequently used as a host for expression of foreign genes because the DNA cloned from higher eukaryotic cells (both cDNAs and genomic clones) is readily expressed since the signals for transcription, mRNA processing, and translation are conserved in higher eukaryotic systems, and the Proteins are expressed in a stable functional form since the machinery to facilitate proper protein folding and assembly are conserved in higher eukaryotic cells. Many post-translational modifications, especially for those proteins that transit the secretory pathway, are efficiently performed, and many proteins are readily secreted from mammalian cells providing the ability to isolate the protein from conditioned medium that contains low amounts of protein when cells are propagated under serum-free conditions.

The choice of a particular expression strategy is dependent upon the objectives of the study. The criteria in evaluating which expression system to employ include: 1. The method desired to introduce the foreign gene into the cell; 2. The particular requirement for a specific cell type in which to obtain expression; 3. The amount of protein expression required to achieve the goals of the study; and 4. The particular need for an inducible vector to obtain expression of proteins that are potentially toxic. Two general methods for transfer of genetic material into mammalian cells are those mediated by virus

infection and those mediated by direct DNA transfer. This chapter will discuss the advantages, utilities, and disadvantages of several vector systems that have proven most successful to obtain high level expression of heterologous genes in cultured mammalian cells. Although there are significant advantages for the use of mammalian cells to express foreign genes, the size of the typical mammalian cell does limit the percentage of total cell protein that can be produced through mammalian cell expression systems.

Expression by Transient DNA Transfection when cells take up DNA, they express it transiently over a period of several days to several weeks (see table 3), and eventually the DNA is lost from the population. The ability to express this DNA over a short period of time is called "transient expression." Transient expression is a convenient and rapid method to study expression of foreign genes in mammalian cells. The efficiency of expression after transient transfection of plasmid DNA is dependent upon the number of cells that incorporate DNA, the gene copy number, and the expression level per gene.

Important features of transient expression systems:			
Extremely short time-frame for the generation of product (days).			
Applicability to a wide range of host cell lines.			
Intrinsic genetic stability and consistency due to extremely short time-frame between generation of vector and product recovered.			
Suitability to multiple processing, allowing study of many genes or mutants at the same time.			
Simplicity, in particular in the construction of expression vectors.			

Table (3): Key features for most of transient expression systems.

The expression level of a gene largely depends upon how efficiently it is transcribed. Transcription of any gene takes place when the RNA polymerase complex interacts with the promoter sequences moves along the gene from a 5¹ to 3¹ direction [15]. This produces an RNA transcript and finally dissociating from the gene at the transcription signal, freezing the transcript for eventual translation. The gene expression in mammalian cells needs a suitable cell line and the appropriate vectors that should act as a vehicle to transport the gene of required interest into the required cell lines.

A number of mammalian cell lines have been utilized for protein expression with the most common being HEK 293 (Human embryonic kidney) and CHO (Chinese hamster ovary). These cell lines can be transfected using polyethyleneimine (PEI) or calcium phosphate [10]. HEK 293 cells exhibit the highest level of PEI-mediated transfection with 50–80% of cells showing green fluorescent protein (GFP) expression, and are now widely used for production of recombinant proteins both by transient

Ma	mmalian cell lines	Mammalian cell lines	
1	HeLa	11	Human embryonic kidney 293 cells
2	HEK293T	12	Recombinant Chinese hemester ovary cell line
3	U2OS	13	MCF-7
4	A549	14	Y79
5	HT1080	15	SO-Rb50
6	CAD,	16	Hep G2
7	P19	17	DUKX-X11
8	NIH 3T3	18	J558L
9	L929	19	Baby hamster kidney (BHK) cells
10	N2a		

transfection as well as by the formation of stable cell lines. A number of mammalian cell lines have been summarized in table (4).

Table (4): A number of mammalian cell lines have been utilized for protein expression.

The main advantages of mammalian cell expression are that the signals for synthesis, processing and secretion of eukaryotic proteins are properly and efficiently recognized by the mammalian cells.

Vectors are autonomously replicating DNA molecules that can be used to carry foreign DNA fragments. It is a vehicle used in gene cloning. DNA of interest is first cloned into an appropriate vector and then by transfection, the gene can be inserted into the host for its expression. For expressing hetrologous genes in mammalian cells, usually vectors derived from mammalian viruses are used. These include viruses such as Simian Viruses 40 (SV40), polyomavirus, herpesvirus and papovirus. Inorder to construct vector the requirement is to select an efficient promoter and also the selection marker.

We used pcDNA5/FRT vector, is a 5.1 kb expression vector designed for use with the Flp-InTM System. We have used his vector becuase it has CMV (Cytomegalovirus) promoter for high level expression in mammalian cells, ten unique restriction site for easy cloning, it has FLP Recombination Target (FRT) site for Flp recombinase-mediated integration of the vector into the Flp-InTM host cell line and Hygromycin resistance gene for selection of stable cell lines. Expression vector is a vector that allows the transcription and translation of a foreign gene inserted into it. Plasmids are circular DNA molecules that lead an independent existence in the bacterial cells. They are naturally occurring, extra chromosomal DNA fragments that are stably inherited from one generation to another generation in an extra chromosomal state. We have the insert of mu opioid receptor tagged with FLAG epitope and yellow fluorescent protein to be transfected in HEK293 cell line.

In most cases, first attempts to transiently express recombinant proteins were executed with 'standard' expression vectors that contain strong viral promoters, such as SV40 or a promoter from cytomegalovirus (CMV).

The choice of the vector depends on the method used for the introduction of the foreign gene into the mammalian cells and on the control elements utilized for the efficient mRNA expression and protein synthesis. There are two general methods for the introduction of foreign DNA into mammalian cells.

Calcium-phosphate, PEI and electroporation have been shown to be useful as vehicles/approaches for large-scale transient gene expression. Commercially available products for DNA transfer are usually sold in small quantities and are not designed to be used in reactors or with large cell masses. Calcium phosphate and PEI achieve DNA transfer by forming complexes with DNA under suitable conditions and these complexes are taken up by cells through endocytosis.

We used polyethylenimine (PEI) method to transfect our recombinant plasmid into HEK 293 cells (see figure 6). Transiently transfected cells express the foreign gene but do not integrate it into their genome. As a consequence the genetic material is not passed from generation to generation during cell division, so the transfected gene will not be replicated and therefore the transiently transfected cells will express the gene for a finite period of time, usually several days.

Before transfection, a sterile high-quality DNA was prepared, (FLAGMOPYFP pcDNA5 (01.7 μ g/ μ l). The vector containing the appropriate expression promoter and the gene of interest should be transformed into a competent bacteria and then the plasmid DNA isolated Commercially available, large-scale plasmid DNA isolated to produce sufficiently high-quality DNA. High-quality DNA is characterized as having an OD260/280 ratio between 1.88 and 1.92, an OD260/230 ratio of 2.1–2.2, and a concentration above 0.5 mg ml⁻¹.



Figure (6): We have seeded HEK293 Cells in 10 cm dishes, after 48 hours we made transfection by PEI method: By mixing 300 μ l of serum free medium, 6 μ l of PEI Solution and 15 μ l of DNA solution of 0.1 μ g/ μ l final concentration and incubating it at room temperature for 10min, then we added the complexes drop wise onto the cells.

CONFOCAL MICROSCOPY OF LIVE CELLS

The development of the first beam-scanning confocal microscope was motivated by the goal of making observations in the tissues of living organisms [16]. The optical sectioning capability of the confocal or multiphoton (MP) microscope allows one to make thin-slice views in intact cells or even intact animals. Confocal microscopes are now fairly common, and because they employ non-ionizing radiation, they are increasingly being used to study living cells and tissue preparations.

For confocal microscopy of living cells, the most important characteristic of the instrument is its efficiency in collecting and detecting the fluorescence emission light from the specimen. Any improvement in this efficiency reduces the amount of light damage and allows the gathering of more data. The increased data can either be in the form of more images, images with less statistical noise, or images obtained with greater spatial or temporal resolution. Newer models of existing commercial confocal microscopes have substantially improved photon efficiency. In addition, there have been technological improvements in the ability to separate the excitation and fluorescence emission of fluorophores, providing greater flexibility for multi-channel imaging and quantitative image analysis in live cells and tissues. Finally, the advantages of either Gaussian-filtering 2D data or deconvolving 3D data to reduce the effects of Poisson noise are now widely appreciated. Routine application of this approach can reduce the light load to the specimen by a factor of from 10 to 100 while still producing images with the same apparent resolution and signal-to-noise.

Confocal images, $(512P\times512 \text{ pixels}; 0.7 \text{ mm pixel size})$ were acquired sequentially on SP5 laser scan microscope (Leica) with a 63×1.4 NA objective, a 2 Airy Units pinhole, $\times7$ electronic zoom and 400 Hz speed using LAS AF acquisition software. Cells were excited sequentially with different laser lines and emission captured between different wavelengths, in order to visualize the same sample with yellow fluorescence protein and Hoechst 33342 reagent. Images were presented after digital adjustment of contrast to maximize signal with images software.

Results

Analysis of Agarose Gel Electrophoresis Images

Protein gel electrophoresis is a simple way to separate proteins prior to downstream detection or analysis. Our portfolio of high-quality protein electrophoresis products unites gels, stains, molecular weight markers and standards, running buffers, and blotting products for your protein analysis experiments. DNA Ladder Standard used in order to determine the size of DNA fragments in an electrophoresis gel. A DNA ladder allows us to make more precise conclusions about our gel electrophoresis results. Since we know the size of each of these bands, we can use them as a reference point for the experimental samples. Consider DNA gel image, by comparing the bands of our sample with the DNA standards of known size, we can conclude that our initial DNA sample was in fact the correct size. Furthermore, we can also conclude that the sample did not contain any detectable nucleic acid contaminants that could disrupt future experimental steps, also, we can conclude if our sample has been digested with a restriction enzyme. Rather than simply conclude that the starting DNA was cut, by using the DNA ladder standards as a point of comparison, we can determine if the DNA was cut in the correct location. In our laboratory we used to take the gel image theoretically in serial cloner program to know the expected number of the bands, and the expected size of each band, and when we get the agarose gel image we compare the result with serial cloner image (Figure 7).

If several samples have been loaded into adjacent wells in the gel, they will run parallel in individual lanes. Depending on the number of different molecules, each lane shows separation of the components from the original mixture as one or more distinct bands, one band per component. Incomplete separation of the components can lead to overlapping bands, or to indistinguishable smears representing multiple unresolved components. Bands in different lanes that end up at the same distance from the top contain molecules that passed through the gel with the same speed, which usually means they are approximately the same size. There are molecular weight size markers available that contain a mixture of molecules of known sizes. If such a marker was run on one lane in the gel parallel to the unknown samples, the bands observed can be compared to those of the unknown in order to determine their size. The distance a band travels is approximately inversely proportional to the logarithm of the size of the molecule.

- Restriction analysis of FLAGMOPYFP Incubated with HindIII + XhoI, resulted in 2 fragments generated (see figure 7.A.1):

1: 1.977 bp, from HindIII [bp number 1] to XhoI [bp number 1978].

2: 6 bp, from XhoI [bp number 1978] to HindIII [bp number 1].

- Restriction analysis of pcDNA5 Incubated with HindIII + XhoI, resulted in 2 fragments generated (see figure 7.A.2):

1: 5.063 bp, from XhoI [bp number 1052] to HindIII [bp number 978].

2: 74 bp, from HindIII [bp number 978] to XhoI [bp number 1052].

- Restriction analysis of FLAGMOPYFPpcDNA5 Incubated with HindIII + XhoI, resulted in 2 fragments generated (see figure 7.B):

1: 5.063 bp, from XhoI [bp number 2955] to HindIII [bp number 978].

2: 1.977 bp, from HindIII [bp number 978] to XhoI [bp number 2955].

- Restriction analysis of FLAGMOPYFP pcDNA5 Incubated with HindIII + ApaI, resulted in 3 fragments generated (see figure 7.C):

1: 5.051 bp, from ApaI [bp number 2967] to HindIII [bp number 978].

2: 1.231 bp, from HindIII [bp number 978] to ApaI [bp number 2209].

3: 758 bp, from ApaI [bp number 2209] to ApaI [bp number 2967].



Figure (7): Analysis of Agarose Gel Electrophoresis Images.

DNA mutation in the sequence of mu opioid receptor is repaired

An indication of the importance of a double-stranded helix to the safe storage of genetic information is that all cells use it; only a few small viruses use single-stranded DNA or RNA as their genetic material. The repair process described before (see figure 5), most of these systems use the undamaged strand of the double helix as a template to repair the damaged strand [17].

The double-helical structure of DNA is ideally suited for repair because it carries two separate copies of all the genetic information, one in each of its two strands. Thus, when one strand is damaged, the complementary strand retains an intact copy of the same information, and this copy is generally used to restore the correct nucleotide sequences to the damaged strand (see figure 8).



Figure (8): After performance of site directed mutagenesis method.

But we found a new gap at position 193, the original Alanine was substituted with by glycine, so we sent the sample with a new primer to check the sequence at that position. The sequencing reaction resulted that there was no mutation at position 193 and all the sequence of our construct FLAGMOPYFPpcDNA5 was correct.

Construction of a recombinant mu opioid receptor tagged with FLAG epitope and yellow fluorescence protein

FLAGMOPYFP insert was successfully ligated to pcDNA5 by HindIII and Xhol digestion as shown (Figure 7. A), the ligated FLAGMOPYFPpcDNA5 was digested by HindIll and Xhol (Figure 7. B), and we found that the fragments were corresponding to the correct expected size.

Digestion of FLAGMOPYFPpcDNA3 and FLAGMOPYFPpcDNA5 by the restriction enzymes Hindlll and Xhol gives the same result in agarose gel image, because both of pcDNA3 and pcDNA5 has the same size approximately, and they share the same restriction sites of Hindlll and Xhol at the same position, the agarose gel image in both of them results in two bands, at 5000 bp and 2000 pb, the first one is the band of the vector and the second one is the band of the insert. So we searched about other

restriction enzymes that give different result in agarose gel image to make sure that our construct was inserted into pcDNA5 vector.

We found that the digestion of FLAGMOPYFPpcDNA3 and FLAGMOPYFPpcDNA5 by the restriction enzyme Xba1 could have a different result in agarose gel image, because the vector pcDNA5 has two restriction sites of Xbal, and the vector pcDNA3 has one restriction site of Xbal (see figure 9).



Firure (9): FLAGMOPYFPpcDNA3 and FLAGMOPYFPpcDNA5 and their restriction enzymes.

So the digestion of FLAGMOPYFP pcDNA5 with Xbal results in two bands and the digestion of FLAGMOPYFPpcDNA3 with Xbal results in one band. We digested our sample with Xbal restriction enzyme, and there were two bands appeared in agarose gel image at the expected sites, so we made sure that our sample was inserted to pcDNA5 vector (see figure 10).



Figure (10): digestion of our sample with Xbal.

Visualization of expression of FLAGMOPYFP receptors in HEK293 living cells by yellow fluorescent protein

YFP was tagged with the construct of MOP for visualization in live cells to identify the positive expression of MOP recombinant in HEK 923 cells. We have assayed the cells about two days after the transient transfection and cells nuclei were stained with Hoechst 33342 reagent in order to visualize them by fluorescence microscopy (see figure 11).





Figure (11): FLAGMOPYFPpcDNA5 visualization by fluorescence microscopy. Confocal images, $(512P \times 512$ pixels; 0.7 mm pixel size) were acquired sequentially on SP5 laser scan microscope (Leica) with a 63×1.4 NA objective, a 2 Airy Units pinhole, $\times 7$ electronic zoom and 400 Hz speed using LAS AF acquisition software.

In this way, yellow fluorescent protein was detected in some cells indicating the presence of a quite good amount of our gene of interest, FLAGMOPYFP, within those cells. We can notice in the images above that not all the cells were expressing FLAGMOPYFP receptors. This is due to the transiently nature of the heterologous expression in this case.

Intracellular distribution of mu opioid receptor might be caused by FLAG tag epitope

HEK 293 cells were transiently transfected with different versions of MOPYFP receptor in different expression plasmids. MOPYFP receptor was tagged with FLAG epitope, a short, hydrophilic protein tag, commonly used in conjunction with antibodies in protein to study protein-protein interactions. The FLAG tag was inserted at the N-terminus, because of its hydrophilic nature, the FLAG tag is commonly found on the surface of a fusion protein, which makes it more available to binding antibodies. We have observed that FLAGMOPYFP receptors are predominantly displaying an intracellular distribution in (see figures 11,A and 11,B), while the distribution of MOPYFP receptor without FLAG tag (see figure 11,C), was essentially in the plasma membrane region. Therefore, we conclude that the presence of FLAG epitope in amino terminus of MOPYFP receptor might be responsible of the different patterns of distribution observed.

The reason behind the intracellular distribution in the cells of FLAG tagged MOP is unknown, but we can think about N-linked glycosylation at N terminus. the attachment of glycan sugar to the nitrogen atom (amide nitrogen) residue of a protein). This type of linkage is important for both the structure and function of some eukaryotic proteins. The N-linked glycosylation process occurs in widely eukaryotes [18]. The nature of N-linked glycans attached to a glycoprotein is determined by the protein and the cell in which it is expressed. It also varies acrossspecies. Different species synthesize different types of N-linked glycan. N-glycan processing is carried out in endoplasmic reticulum (ER) and the Golgi body. Initial trimming of the precursor molecule occurs in the ER and the subsequent processing occurs in the Golgi. Upon transferring the completed glycan onto the nascent polypeptide, three glucose residues are removed from the structure. Enzymes known as glycosidases remove some sugar residues. These enzymes can break glycosidic linkages by using a water molecule. These enzymes are exoglycosidases as they only work on monosaccharide residues located at the non-reducing end of the glycan.

Once the protein is folded correctly, the three glucose residues are removed by glucosidase I and II. The removal of the final glucose residue signals that the glycoprotein is ready for transit from the ER to the cis Golgi. However, if the protein is not folded properly, the glucose residues are not removed and thus the glycoprotein can't leave the endoplasmic reticulum. A chaperone protein (calnexin/calreticulin) binds to the unfolded or partially folded protein to assist protein folding.



(A) MOPYFP receptor tagged with FLAG epitope and subcloned into pcDNA5 vector.

- A.1 Cells nuclei of the transfected cells.
- A.2 Yellow fluorescent protein expression in the transfected cells.
- A.3 Cells nuclei merge with Yellow fluorescent protein expression in the transfected cells.



(B) MOPYFP receptor tagged with FLAG epitope and subcloned into pcDNA3 vector.

B.1 Cells nuclei of the transfected cells.

B.2 Yellow fluorescent protein expression in the transfected cells.

B.3 Cells nuclei with Yellow fluorescent protein expression in the transfected cells.



(C) MOPYFP receptor untagged with FLAG epitope and subcloned into p-eYFP vector. C.1 Cells nuclei of the transfected cells.

- $\label{eq:c.2} C.2 \ \mbox{Yellow fluorescence protein expression in the transfected cells}.$
- C.3 Cells nuclei with Yellow fluorescent protein expression in the transfected cells.

Firure (11): FLAGMOPYFPpcDNA5, FLAGMOPYFPpcDNA3 and MOPYFP p-eYFP visualization by fluorescence microscopy.

Discussion

Despite of the fruitful history of GPCRs in the drug development industry, discovering or designing new therapeutic agents that modulate a GPCR in a specific way is still a challenge, and in fact is undergoing a renewal because of explosion of new knowledge about their structure and ways to more precisely control their signaling via which G protein or other receptor-associated protein they couple to (biased signaling). Mu opioid Receptors are the most common behavioral function linked to GPCR have been their ability to mediate analgesic effects.

For thousands of years mu opioid agonists such as morphine have been utilized for their analgesic properties. Today, morphine and related compounds are still used as a first line therapy in the treatment of moderate to severe pain [19]. However, despite the clear benefits of mu agonists in pain management, severe side effects such as dependence and respiratory depression are associated with use of these drugs. To date, there are only two approved mu opioid antagonists for use in the treatment of these adverse effects, that is, naloxone and naltrexone. However, many other clinical and therapeutic areas have been linked to mu opioid receptor antagonism. These include treatment of opioid induced pruritus of the skin, obesity, and Parkinson-induced tardive dyskinesia. Currently there are two compounds, N-methylnaltrexone and alvimopan, under FDA review as possible treatments for opioid induced bowel dysfunction and postoperative ileus. These compounds are of special interest as they are peripherally restricted. This attribute enables treatment of peripheral side effects induced by opioid agonists without reversal of the centrally mediated analgesia of the agonist. In this article we discuss the structural classes of mu opioid antagonists, their potential clinical applications, and review the relevant patents of the last ten years.

There is a clear interest for heterologous expression systems that can provide the possibility to express genetically modified receptors to conduct molecular pharmacology investigations. To meet this interest, the main purpose of our research was the construction of a recombinant mu opioid receptor tagged with FLAG epitope and fused to YFP to generate an inducible FLIP-In HEK 293 stable cell line to conduct further investigations on its molecular pharmacology.

The expression of cloned genes in mammalian cells is a basic tool for understanding gene expression, protein structure, and function, and biological regulatory mechanisms. The level of protein expression from heterologous genes introduced into mammlaian cells depends upon multiple factors including DNA copy number, efficiency of transportation, mRNA processing, mRNA transport, mRNA stability, and translational efficiency, and protein processing, transport, and stability. Different genes exhibit different rate limiting steps for efficient expression. Multiple strategies are available to obtain high level expression in mammalian cells. Two general methods for transfer of genetic material into mammalian cells are those mediated by virus infection and those mediated by direct DNA transfer. Although there are significant advantages for the use of mammalian cells to express foreign genes, the size of the typical mammalian cell does limit the percentage of total cell protein that can be produced through mammalian cell expression systems. When cells take up DNA, they express it transiently over

a period of several days to several weeks and eventually the DNA is lost from the population. The ability to express this DNA over a short period of time is a convenient method to study expression of foreign genes in mammalian cells.

Conclusion

The opioid receptors belong to the G-protein-coupled receptor (GPCR) superfamily. The mu opioid receptor (MOP) is a member of the G protein-coupled receptor family and constitutes the main target of endogenous opioid neuropeptides and morphine. MOP activation by agonist compounds has a wide variety of pharmacological and physiological effects involved in addiction, pain treatment and others. Therefore our understanding of MOP receptor function is central to the development of different therapies.

In conclusion, we successfully constructed the recombinant eukaryotic expression plasmid containing mu opioid receptor gene was effectively expressed in human embryonic kidney 293 (HEK) cells. By tagging a FLAG epitope at amino terminus and a yellow fluorescent protein (YFP) at carboxyl terminus, subsequently FLAGMOPYFP receptor was subcloned into pcDNA5/FRT/TO vector to generate a future inducible FLIP-In HEK 293 stable cell line. Repair of DNA mutation in FLAGMOPYFP was an essential process in this project, because the DNA is repository of genetic material in each living cell and its integrity is essential for viability of our construct.

To observe the cellular expression of FLAGMOPYFP in fluorescence microscopy, HEK 293 cells were transiently transfected with this chimeric MOP receptor subcloned in different expression plasmids, i.e. pcDNA3 and pcDNA5 vectors. Additionally, an amino terminus untagged form of MOPYFP receptor was also visualized to compare with the FLAGMOPYFP version. Finally, we concluded that the presence of FLAG epitope in amino terminus of MOPYFP receptor might be responsible of the different patterns of distribution observed.

An intriguing result of our investigations was the different pattern of distribution observed when comparing the cellular expression of FLAGMOPYFP and MOPYFP that opens new avenues of research to further explore the reason behind this result.

Acknowledgments

I would like to thank Dr. Juan Francisco López-Giménez for his efforts. I also thank my partner Carolina Del Cerro and our laboratory group for assistance. I am eternally grateful to my parents for their endless patience and never ending knowledge. I am thankful for my husband for being helpful and for encouraging me to do my best. Finally I thank all my family and my second family in Santander for their encouragement and support.

References

- Brian K. Kobilka, 2007. G Protein Coupled Receptor Structure and Activation, Biochim Biophys Acta. 2007 Apr; 1768(4): 794–807.
- Li YY, Hou TJ, 2010. Computational modeling of structure-function of g protein-coupled receptors with applications for drug design, Curr Med Chem 17(12):1167-80.
- John A. Salon, David T. Lodowski, and Krzysztof Palczewski, 2011. The Significance of G Protein-Coupled Receptor Crystallography for Drug Discovery, Pharmacol Rev. 2011 Dec; 63(4): 901–937.
- Trzaskowski, B; Latek, D; Yuan, S; Ghoshdastider, U; Debinski, A; Filipek, S 2012. Action of molecular switches in GPCRs theoretical and experimental studies, Current medicinal chemistry 19 (8): 1090–109.
- B Trzaskowski, D Latek, S Yuan, U Ghoshdastider, A Debinski, and S Filipek, 2012. Action of Molecular Switches in GPCRs - Theoretical and Experimental Studies, Curr Med Chem. 2012 Mar; 19(8): 1090–1109.
- Stephen J. Hill, 2006. G-protein-coupled receptors: past, present and future Institute of Cell Signalling, British Journal of Pharmacology (2006) 147, S27–S37.
- Ream Al-Hasani, and Michael R. Bruchas, 2013. Molecular Mechanisms of Opioid Receptor-Dependent Signaling and Behavior, Anesthesiology. 2011 Dec; 115(6): 1363–1381.
- 8. Karim Nagi and Graciela Piñeyro, 2011. Regulation of opioid receptor signalling: Implications for the development of analgesic tolerance, Mol Brain. 2011; 4: 25.
- Juan F. Lopez-Gimenez, M. Teresa Vilaró and Graeme Milligan, 2008. Morphine Desensitization, Internalization, and Down-Regulation of the μ Opioid Receptor Is Facilitated by Serotonin 5-Hydroxytryptamine_{2A} Receptor Coactivation, Molecular Pharmacology, 51278-1291.
- Kaufman RJ, 2000. Overview of vector design for mammalian gene expression, Mol Biotechnol. 2000 Oct;16(2):151-60.
- Kishwar Hayat Khan, 2013. Gene Expression in Mammalian Cells and its Applications, Adv Pharm Bull. 2013 Dec; 3(2): 257–263.
- Tsien, Roger Y.; Llopis, Atsushi; Heim, Juan; McCaffery, Roger; Adams, J. Michael; Ikura, Joseph A.; Tsien, Mitsuhiko (1997). "Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin". Nature 388 (6645): 882–7.
- Takeharu Nagai, Keiji Ibata, Eun Sun Park, Mie Kubota, Katsuhiko Mikoshiba & Atsushi Miyawaki, 2002. A variant of yellow fluorescent protein with fast and efficient maturation for cellbiological applications, Nature Biotechnology 20, 87 - 90 (2002).
- Berg JM, Tymoczko JL, Stryer L, 2002. Mutations Involve Changes in the Base Sequence of DNA, Section 27.6, Biochemistry. 5th edition, New York: W H Freeman; 2002.
- 15. Kishwar Hayat Khan, 2013. Gene expression in mammalian cell lines.
- Pawley JB (editor) (2006). Handbook of Biological Confocal Microscopy (3rd ed.). Berlin: Springer. ISBN 0-387-25921X.

- 17. Alberts B, Johnson A, Lewis J, et al, 2002. Molecular Biology of the Cell. 4th edition, New York: Garland Science; 2002.
- Patterson MC (2005). "Metabolic mimics: the disorders of N-linked glycosylation". Semin Pediatr Neurol 12 (3): 144–51. doi:10.1016/j.spen.2005.10.002. PMID 16584073.
- 19. Goodman AJ¹, Le Bourdonnec B, Dolle RE, 2007. Mu opioid receptor antagonists: recent developments, ChemMedChem. 2007 Nov; 2(11):1552-70.