http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 8 (8): 1115-1118, 2005
ISSN 1028-8880

N53Q Site Directed Mutagenesis of Rat Mu Opioid Receptor and its Cloning Using pcDNA3 Vector

M. Rabbani, H. M. Sadeghi, A. Jafarian-Dehkordi, V. Hajhashemi and A. Rostami
Department of Pharmacology, School of Pharmacy and Pharmaceutical Sciences,
Isfahan University of Medical Sciences, Isfahan, Iran

Abstract: Mu Opioid receptor plays an important role in mediating most of diverse effects of opioids like analgesia, euphoria and dependence. Studies on Glycosylation Consensus of various receptors showed that it has important roles in the affinity, desensitization, G-protein coupling and intracellular signaling. The aim of the present study was to create a mutation in this consensus of Rat Mu Opioid Receptor (RMOR) for further investigation of its function. Using nested PCR one mutation was produced, N53Q. The PCR products as well as the pcDNA3 vector were digested using *Hind*III and *BamH*I restriction enzymes and were ligated and transformed to Ecoli HB101 cells. The obtained colonies were analyzed for the presence of the inserts using suitable restriction enzymes. The obtained plasmids are ready for further investigations in binding assay and function assays like cyclic AMP measurements.

Key words: Mutation, Rat Mu Opioid Receptor, nested PCR

INTRODUCTION

Opioids exert their effects by stimulation of the four main opioid receptors: Mu, delta, kappa and recently discovered N/OFQ[1]. Activation of mu opioid receptor can elicit most typical opioid effects, e.g. analgesia, rewarding, respiratory depression, gastrointestinal effects, neuro-hormonal changes, tolerance and dependence[1]. Glycosylation is one of the most important post-translational modifications that may exert prominent imprints in the receptor proteins. Previous studies have revealed that receptor glycosylation can have profound effects on affinity[2], desensitization[3], function[4], G-protein coupling[5] and signal transduction[6].

Glycosylation can occur in all amino acid sequences in the form of N.X.T or S that is glycosylation consensus[7]. Mu opioid receptor has some of that consensus on its N-terminal. To our knowledge only two studies performed on the role of glycosylation in human Mu opioid receptor one of them had shown that N40D substitution could cause an increase in affinity and potency of ligand in activation of potassium channels[8]. In contrary, the second study indicates that the same mutation (N40D) had no difference in affinity, petency, Signaling and also down regulation in comparison to wild type. In the present study, N53Q substitution on Rat Mu Opioid Receptor (RMOR) was constructed, as the N53 in RMOR is the closest of the glycosylation site to that of the human. N (Asparagine) and Q (Glutamine) are constitutionally similar amino acids. By performing N53Q mutation, the glycosylation site of the RMOR will be interrupted, but the rest of the receptor characteristics would be preserved.

MATERIALS AND METHODS

Materials: 10x PCR buffer and DNA Taq polymerase enzyme were from Bio-tools, dNTP was from Eurobio, restriction enzymes were from Roche company, DNA ligase enzyme, buffers and DNA weight marker were from Fermentas, plasmid preparation kit were from Bio-Rad, gel extraction kit was from Qiagen. The RMOR cDNA was kindly provided by Professor G. Henderson (Bristol, UK).

Methods

Primer design: Using WDNASIS program, a pair of primers were designed for N53Q (asparagines to glutamine) mutant. The following oligonucleotide primers were used for preparing mutant on RMOR: Forward (sense) 5′GCTTGGCGGGCAAGACAGCCTGTG3′ and reverse (anti-sense) 5′CGAACCGCCCGTTCTGTCGGACAC3′. Mutagenesis primer N53Q corresponds to 24 nucleotides 146 to 170 of the coding sequence of RMOR. Mismatch at

Corresponding Author: Dr. M. Rabbani, Department of Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran
Tel: +98 311 7922646 Fax: +98 311 6680011

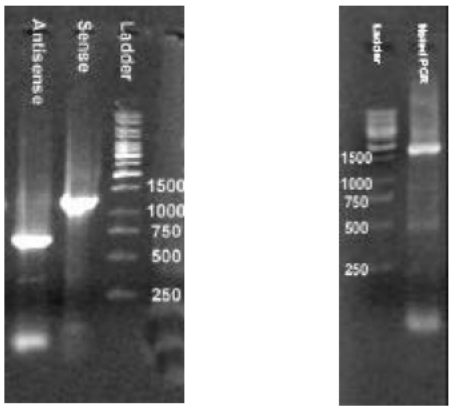
position 159 led to the replacement of Asparagine by Glutamine at amino acid position 53. Mutation was confirmed by double-strand DNA sequencing. Outer pcDNA3 primers: sense (5′TGGGTGGACTATTTACGG3′) and anti-sense (5′AGACTCCGCCTTTCTTG3′). Inner pcDNA3 primers: sense (5′GTGGATAGCGGTTTGACT3′), anti-sense (5′GGCACGGAAGGAACTGG′)

Nested PCR: A mixture of cDNA of RMOR (as template), 10x PCR buffer, dNTP, DNA Taq polymerase, sense N53Q and anti-sense outer pcDNA3 primers in one eppendorf tube (total volume of 50 μL) was set in thermocycler for preparation of PCR products. Another mixture was prepared using antisence N53Q and sense outer pcDNA3 together with pcDNA3 of RMOR (as template), 10xPCR buffer, dNTP and DNA Taq polymease (Total volume of 50 μL). Protocol of PCR was set as following: 1 cycle (94°C for 5 min), 33 cycles (94°C for 1 min, 55°C for 2 min, 72°C for 2 min) and 1 cycle (72°C for 20 min) these two tubes were our first PCR products. The PCR products were run on agrose gel and detected their size. In the second (nested) PCR, a mixture of products of first PCR (as template), 10x buffer of PCR, dNTP and DNA Taq polymerase and of each sense and anti-sense inner pcDNA3 primers (total volume of 50 μL) was set as above[9,10].

Ligation and transformation: The PCR product was run on a 0.7% agarose gel containing ethidium bromide and then was visualized and photographed using a transilluminator and gel documentation system. All digestions were performed using the suitable restriction enzymes at 37°C for 1 h. A DNA extraction kit was utilized for isolation of the DNA bands from the agarose gels. The vector and insert (molar ratio of 1/3, respectively), DNA ligase and its buffer were added at a final volume of 15 μL and were incubated at 16°C overnight. The product of ligation was transformed using HB101 competent cells. The heat shock was performed for 1 min at 42°C. The cells were spread over LB plates containing ampicillin and were incubated overnight at 37°C. The obtained colonies were cultured in LB medium at 37°C overnight. Plasmid preparation was carried out using plasmid preparation kit.

RESULTS AND DISCUSSION

Using the sense primer in RMOR containing mutation sequence and anti-sense outer pcDNA3 primer a sharp band was observed between 1000 and 1500 bp (Sense lane, Fig. 1a). Anti-sense primer in RMOR containing mutation sequence and sense outer pcDNA3 primer produced a sharp and dense band between 500 and



(a) (b)

Fig. 1: Gel electrophoresis of first and second PCR sets. (a) First PCR set of N53Q mutant at RMOR. Sense and anti-sense segments are shown. (b) Second (Nested) PCR set of N53Q mutant at RMOR. 0.7 percent agarose gel was used

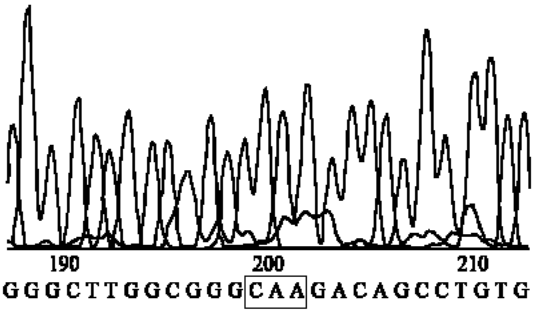
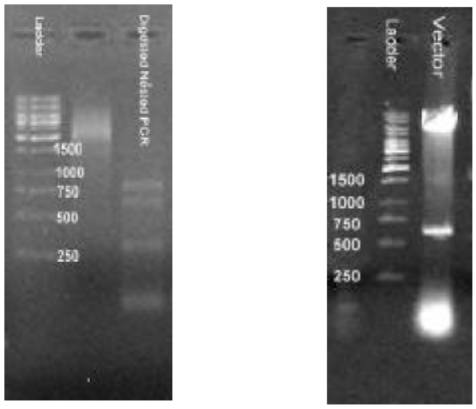


Fig. 2: Sequencing of nested PCR product. Selected part of nested PCR sequencing showing embedded mutation in RMOR sequence (Black Rectangle). AAC in wild RMOR cDNA have converted to CAA

750 bp (Anti-sense lane, Fig. 1a). The first PCR products together with the inner sense and anti-sense pcDNA3 primers, resulted in a band lying between 1500 and 2000 bp (Nested PCR lane, Fig. 1b)

The second PCR containing the desired mutation were used for the digestion, sequencing and subcloning into the pcDNA3 vector. Sequencing of the nested PCR product confirms the mutation insertion (Fig. 2).

The pcDNA3 vector containing the wild type RMOR receptor as well as the nested PCR product were cut by *Hind*III and *Bam*HI restriction enzymes for obtaining vectors and inserts (Fig. 3a and b). The digestion product of the vector containing the wild type RMOR using the above restriction enzymes produced a band of approximately 500 bp and the remaining band at about



(a) (b)

Fig. 3: Digestion of RMOR containing pcDNA3 and nested PCR product for extraction of vector and insert. (a) Digestion of pcDNA3 containing wild RMOR (Vector) using *Hind*III and *BamH*I restriction enzymes. (b) digestion of Nested PCR product by *Hind*III and *BamH*I restriction enzymes. 0.7% agarose gel was used

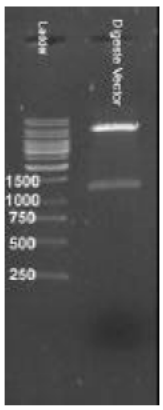


Fig. 4: Digestion of transformed vector containing mutated RMOR cDNA. The segments appeared by digestion of recombinant vector with *Hind*III and *Xba*I confirmed insertion of mutated region into the vector (Digested Vector). 0.7% agarose gel was used

6 kb that was extracted from the gel (Fig. 3a). The nested PCR product that was digested with *Hind*III and *Bam*HI restriction enzymes produced the required band about 500 bp and two other bands, as expected (Fig. 3b). The 500 bp piece DNA of digested nested PCR product that was extracted from the gel was ligated into the extracted segment of the plasmid. The ligation product was transformed to HB101 bacteria using heat shock method. Plasmid preparation was performed on the resultant colonies. The presences of the inserts were confirmed by the *Hind*III and *Xba*I digestion (Fig. 4). This digestion resulted in a band about 1.2 kb and the remaining vector about 5.5 kb.

In order to study the role of glycosylation consensus in the function of opioid receptors, we created the N53Q mutant in RMOR. Using the mutant primers in the PCR reaction, the expected size bands were obtained. The results of the sequencing confirmed, that the DNA was mutated in the right region. The restriction analysis of the PCR fragments that contained the mutant DNA, further proved the integrity of the DNA. Using suitable restriction enzymes the appropriate pieces of insert and vector were extracted and ligated together. Restriction analysis of the ligated fragments confirmed that the mutated DNA fragment has indeed inserted into the vector with the right orientation. The plasmid construct is now ready to be used for further functional and binding assays.

ACKNOWLEDGMENTS

This project was carried by MUI grant. We would also like to thank Mrs. Moazen for her assistance in this project.

REFERENCES

1. Gutstein, H.B. and H. Akil, 2001. Opioid analgesics. In: Hardman, J.G., L.E. Limbird (Eds.) Goodman and Gilman's The Pharmacological Basis of Therapeutics. New York: McGraw-Hill, pp: 569-587.
2. Akiyama, S.K., S.S. Yamada and K. M. Yamada, 1989. Analysis of the role of glycosylation of the human fibronectin receptor. J. Biol. Chem., 264: 18011-18018.
3. Nishizaki, T., 2003. N-glycosylation sites on the nicotinic ACh receptor subunits regulate receptor channel desensitization and conductance. Brain Res. Mol. Brain Res., 114: 172-176.
4. Saito, Y., M. Tetsuka, L. Yue, Y. Kawamura and K. Maruyama, 2003. Functional role of N-linked glycosylation on the rat melanin-concentrating hormone receptor 1. FEBS Lett., 533: 29-34.
5. Benya, R.V., T. Kusui, T. Katsuno, T. Tsuda, S.A. Mantey and J.F. Battey *et al*., 2000. Glycosylation of the gastrin-releasing peptide receptor and its effect on expression, G protein coupling and receptor modulatory processes. Mol. Pharmacol., 58: 1490-1501.

6. Buteau, H., A. Pezet, F. Ferrag, M. Perrot-Applanat, P.A. Kelly and M. Edery, 1998. N-glycosylation of the prolactin receptor is not required for activation of gene transcription but is crucial for its cell surface targeting. Mol. Endocrinol., 12: 544-555.
7. Apweiler, R., H. Hermjakob and N. Sharon, 1999. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim. Biophys. Acta, 1473: 4-8.
8. Bond, C., K.S. LaForge, M. Tian, D. Melia, S. Zhang and L. Borg *et al.*, 1998. Single-nucleotide polymorphism in the human mu opioid receptor gene alters beta-endorphin binding and activity: Possible implications for opiate addiction. Proc. Natl. Acad. Sci. USA., 95: 9608-9613.
9. Befort, K., D. Fillol, F.M. Decaillot, M.R. Gaverianx-Rutts, Hoehe and B.L. Kieffer, 2001. Asingle nucleotide porlymorphic mutation the human Mu-opioid receptor impair receptor Signaling. J. Biol. Chem., 276: 3130-3137
10. G. Innamorati and M. Birnbaumer, 1997. Biochemical basis of partial nephrogenic diabetes insipidus phenotypes. Mol. Endocrinol., 11: 1806-1813.