cDNA-Derived Amino Acid Sequence from Rat Brain $A_{2a}R$ Possesses Conserved Motifs PMNYM of TM 5 Domain, Which May Be Involved in Dimerization of $A_{2a}R$

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Abstract. The human adenosine A_{2a} receptor ($A_{2a}R$) belongs to the family of G-protein coupled receptors (GPCRs), characterized by seven transmembrane (TM) helices. TMs are involved in various cellular processes including dimerization-mediated recognition of ligand. TM5 has been suggested to self associate and may be involved in the dimerization of $A_{2a}R$. However the role of dimerization and the motifs involved in dimerization of TM 5 have not been revealed. To study the folding and assembly of $A_{2a}R$, the cDNA of the adenosine $A_{2a}R$ from rat brain was isolated and sequenced (DQ098650). The computational analysis (gi|70727927|gb|AAZ07991.1|) showed that the protein of 42 amino acid residues aligned in TM 5 domain region of AA2AR_RAT (P30543). PROSITE search illustrated that the motif PMNYM was conserved in $A_{2a}R$ and the motif PMSYM was present in $A_{2b}R$ respectively. The minimal dimerization motif in the TM 5 domain of the rat A_{2a} receptor sequence DQ098650 has found to be the motif PXXXM/Y.

Keywords: Adenosine A_{2a} receptor, cDNA, GPCRs, RT-PCR, TM 5.

1 Introduction

Adenosine A_{2a} receptors have a localized distribution and have emerged as a promising drug target for treating many neurological and psychiatric disorders such as Parkinson's disease [4,5,6,7], schizophrenia and affective disorders [8,9,10]. The adenosine A_{2a} receptors ($A_{2a}R$) belong to the G-protein coupled receptor (GPCR) super family characterized by seven transmembrane (TM) helices arbitrating a surfeit of signals across the plasma membrane in the cell modulating many physiological processes [11,12,13]. Assembly of transmembrane (TM) domains is a critical step in the function of membrane proteins. The determinants of transmembrane receptors structure, folding, assembly, activation mechanism and oligomeric states to function as monomers, dimers, or larger oligomers are wrapped. The existence of GPCRs as homodimers, heterodimers, or even as higher order oligomers [14, 15, 16, 17] assist in

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GPCRs' functions, including ligand binding, receptor activation, desensitization, and trafficking, as well as receptor signaling [18, 19, 20]. The essential residues required for recognition of adenosine receptor agonists and/or antagonists binding within the transmembrane helical domains (TMs) 3, 5, 6, and 7, coincide largely with the corresponding amino acids of the binding site of cis-retinal in rhodopsin, although there are additional interaction sites within TMs 6 and 7 of the ARs in comparison with the binding site of rhodopsin [21]. Essentially, the constitutive and ligand-induced oligomerization has been established in other receptors [22]. However mechanism of intermolecular interaction remains unclear. A five residue motif (GxxxG) responsible for specific homodimerization for TM helices of a bioptic membrane protein have been reported. The GxxxG motif present in TM 1 of yeast α-factor is essential in oligomerization. Polar clamps and serine zipper motifs have also been identified. Since GPCRs may not have similar structures due to differences in helix-orientation, helix-helix interactions different mechanism of folding may exist in GPCR [23]. The ability of TM in oligomerization and dimerization has been reported recently and the involvement of the five residue PxxxM pattern have been suggested in dimerization of $A_{2a}R$ [24]. To study the mechanism of ligand interaction to the $A_{2a}R$, we implicated our attention to understand the determinants of $A_{2a}R$ folding and assembly.

In the present study, a primer set specific for adenosine A_{2a} receptor gene encoding TM 5 domain region have been designed. A partially amplified cDNA fragment of approx. 127bp was obtained by RT-PCR and sequenced (NCBI Gen Bank database accession no. DQ098650). Computation of sequence analysis showed that the rat brain adenosine A_{2a} receptor protein of 42 amino acid residues (gi|70727927|gb|AAZ07991.1|) aligned in TM 5 domain region of AA2AR_RAT (P30543) and exhibited 85.17% homology (36 amino acid residues) with TM 5 domain region of AA2AR_HUMAN (P29274). We carried the PROSITE search of PMNYMV residues present in the submitted sequence with the known amino acid sequences of mammalian adenosine receptors. We found that PMNYM motif was conserved in the TM 5 region of all mammalians $A_{2a}R$ and PMSYM motif was present in TM 5 region of all mammalians $A_{2b}R$ [25], thus suggesting a general role of these patterns/motifs in TM assembly. This is the first reported evidence of showing the presence of conserved motifs PMNYM in $A_{2a}R$ and PMSYM in $A_{2b}R$ respectively, which may be involved in transmembrane domain self-association, and lays ground for more apprehended analysis of adenosine receptors dimerization. The dimerization of $A_{2a}R$ involving PMNYM motif present in TM 5 is focus of our further investigations employing *in silico* and *in vitro* experimental studies.

2 Materials and Methods

2.1 Dissection and Isolation of Rat Brain Striatal Tissues

Adult wistar rats (~250g from animal house facility at Dr. B.R. Ambedkar Center for Biomedical Research, India) were sacrificed by cervical dislocation and decapitated. The freshly collected skull was cut-opened from the dorsal side. Whole brain was immediately placed in a sterilized glass petridish containing ice-cold PBS (pH 7.4)

and striatal tissue was dissected out from the mid-brain region. Striatal tissue was processed immediately for the RNA extraction [26].

2.2 Total RNA Isolation

Total striatal RNA was extracted by TRIzol reagent method [27]. Briefly, tissue (100-200mg) was rinsed in cold PBS buffer (137mM NaCl, 2.7mM KCl, 4.3mM Na2HPO4, 1.4mM KH2PO4, pH 7.5), placed in 1 ml of Trizol reagent (0.8M Guanidine thiocyanate, 0.4M Ammonium thiocyanate, 0.1M Sodium acetate pH 5.0, 38% Saturated phenol and 5% Glycerol) and immediately homogenized with a glass homogenizer to quickly dissociate the tissue and incubated at room temperature for 3 minutes. About 200µl of the chloroform (Sigma-Aldrich, USA) was added to the tube, mixed gently and incubated at room temperature for 10 minutes. The sample was centrifuged at 13,000 rpm for 15 minutes at 4^0C. Clear supernatant containing total RNA and trace amount of DNA was transferred to a fresh eppendorf tube and was precipitated by adding chilled isopropanol (at -20^0C) (Sigma-Aldrich, USA). The tube was kept at -20^0C for 30 minutes and centrifuged at 13,000 rpm for 10 minutes at 4^0C. RNA pellet was washed twice with ice-cold 70% ethanol and air dried for 30 minutes. Pellet was resuspended in 20µl of Diethylpyrocarbonate (DEPC)-treated water. All solutions were prepared from DEPC-treated autoclaved, distilled water.

2.3 DNase I Treatment

To the tube containing total RNA, 2 µl of 10X DNase buffer (0.5M Tris-HCl pH 7.5, 0.5mg/ml BSA) and 2 µl of 10 U/µl DNase I (Sigma-aldrich; 20 units total) was added and incubated at 37°C for 2 hours. RNA was re-extracted by adding: 2 µl of 2M sodium acetate, pH 4.0, 22 µl water saturated phenol and 6 µl Chloroform-isoamyl alcohol. vortexed vigorously for 15 seconds. Placed on wet ice for 15 minutes and centrifuged for 10 minutes at 4°C. Upper layer was transferred to fresh tube [28].

2.4 mRNA Purification and Quantitation

Striatal mRNA was extracted by Nucleotrap mRNA mini purification kit (BD Biosciences, USA) by using manufacturer's protocol. Purified mRNA was quantitated by using UV/vis. Spectrophotometer (Schimadzu Corp, Kyoto, Japan) [29].

2.5 Primer Designing

Specific primers were designed based on the TM 5 sequence of the adenosine A_{2a} receptor DNA from *Rattus norvegicus* (30). Primer 3 (online software for primer designing) was used to evaluate secondary priming sites and inter and intra-primer complementation (31).

2.6 RT-PCR Amplification

Reverse transcriptase (RT)-PCR was performed on the purified mRNA using a Titanium One-Step RT-PCR kit (Clontech/BD Biosciences, Palo Alto, CA) according to the manufacturer's protocol. A forward oligo DNA primer with sequence (5'-CCA

TGC TGG GCT GGA ACA-3'), a reverse oligo DNA primer with sequence (5' – GAA GCG GCA GTA ACA CGA ACG-3') and an oligo (dT)17 primer were used in the RT-PCR reaction at a concentration of 45 µmol/L (primers synthesized by Microsynth, Switzerland). A mouse ß-actin primer (Clontech) served as the positive control. The RT-PCR reaction and amplification were performed under the following conditions: 1 hour (50°C), 5 minutes (94°C), 30 times (92°C 1 minute, 55°C 1 minute, 72°C 1 minute), 2 minutes (68°C). The amplified RT-PCR product was then subjected to electrophoresis on a 1.5% agarose gel (0.5 µg/ml ethidium bromide) for 1.5 hours at 80 V. The gel was visualized using Alpha Imager 1220 documentation and analysis system (Alpha Innotech Corporation, San Leandro, California). The PCR product (approx. 150bp fragment) was eluted from the low melting agarose and purified by Genei quick PCR purification kit (Bangalore genei, India). Purified fragments were lyophilized and sent to Microsynth, Switzerland for sequencing.

2.7 Sequence Analysis

Nucleotide sequence of the cDNA fragment was analyzed by Nucleotide-nucleotide BLAST (blastn) to find out cDNA fragment homology with all organism database and was submitted online to NCBI GenBank database [32]. Further, PSI-BLAST was carried on SWISS Prot Data Base for determining the positions of conserved amino acids using GAP extension 7/2 having opening penalty 7 and extension penalty 2 [33]. Multiple sequence alignment was done by Clustal-X (Version 1.83) to find out sequence homology to human and rat brain adenosine A_{2a} receptor TM 5 domain with submitted sequence (accession no. AAZ07991.1). PROSITE search was carried to find out conserved motif.

3 Results and Discussion

3.1 RT-PCR Amplification of Adenosine A_{2a} Receptor cDNA Encoding TM 5 Domain

The TM4 and TM5 have been shown to be involved in intradimeric contact in oligomeric molecular model of rhodopsin [34, 35, 36] while TM1, TM2, and the cytoplasmic loop connecting TM5 and TM6, facilitate the formation of rhodopsin dimer rows. Two conserved serine residues in TM 5 postulated to be part of a ligand-binding site in the adrenergic receptor [37]. The amino acid sequence encoded by cDNA fragment from rat brain contained conserved sequences which had characteristics of the G-linked class of receptors and displayed sequence homology in TM 5 domains with the human A_{2a} receptors (85%). We designed highly specific primer set by considering the exon sequence of adenosine A_{2a} receptor gene to isolate the cDNA fragment encoding TM 5 domain of rat brain adenosine A_{2a} receptor using Primer3 primer design tool (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [38].). Rat brain striatal RNA was isolated using TRIzol reagent method and was depicted by 1% agarose gel electrophoresis (Fig.1). A desired cDNA fragment of approximately 150bp was obtained by RT-PCR

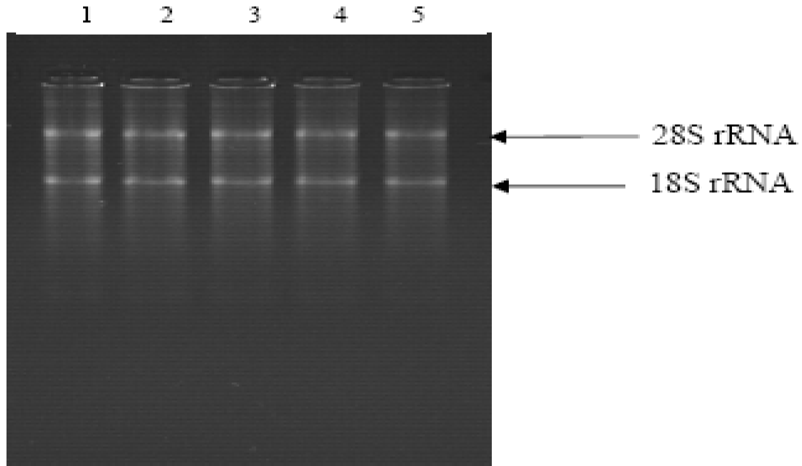

Fig. 1. Total rat brain striatal RNA. TRIzol reagent was used to isolate the total RNA . Lane 1-5 represents two distinct bands of 28S rRNA and 18S rRNA.

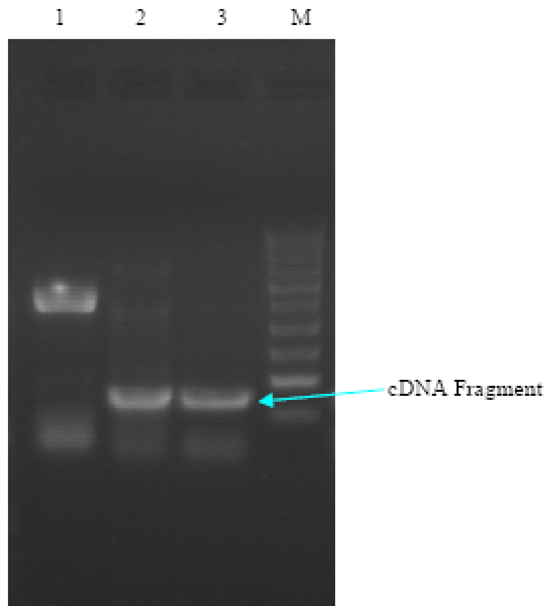

Fig. 2. RT-PCR products. Single stranded cDNA was synthesized from 1µg of total poly $(A)^+$ striatal RNA using MMuLV-RTase. Lane 1. Control (mouse β-actin cDNA product, 540bp), Lane 2 & 3. Amplified cDNA fragment of approx. 150bp, Lane 4. 100bp DNA marker, Light faint bands below 100bp represents non-specific cDNA fragments.

amplification of purified rat brain mRNA (Lane 2 & 3, Fig. 2). Mouse β-actin primer set was used as control to obtain a cDNA product of 540bp (Lane 1.). Approximate size of the amplified cDNA fragment was illustrated with the aid of a 100bp DNA ladder. Faint bands below 100bp represent the non-specific fragments or primer dimers. The purified cDNA fragments possessed 127bp length on sequencing (Microsynth, Switzerland). The sequence was submitted to the GenBank database (Accession No. DQ098650, Dated: 19 Jun, 2005).

3.2 Sequence Analysis of Rat Brain Adenosine A_{2a} Receptor cDNA

In order to enable mechanistic understanding of A_{2a}R, computational studies were carried to predict the possible interaction interfaces. We conceded the evolutionary relation existing among the adenosine receptors in terms of their sequences that is measurable in common elements of their structural and functional features. Sequence analysis of amplified rat adenosine A_{2a} receptor cDNA was carried by Nucleotide-nucleotide BLAST (blastn) with all organisms gene database. We found a total of 69 blast hits and it was found that rat (*Rattus norvevicus*) adenosine receptor mRNA exhibited 90% homology (Score = 178 bits (90), Expect = 3e-42 Identities = 96/98 (97%), Gaps = 0/98 (0%) with the isolated cDNA sequence. Besides, it has also been observed that mouse (*Mus musculus*) strain C57BL/6J clone rp23-288i20 showed 68% homology (Score = 135 bits (68), Expect = 4e-29 Identities = 89/96 (92%), Gaps = 0/96 (0%)), Homo sapiens ADORA2A, mRNA (cDNA clone IMAGE:100000002) showed 34% homology (Score = 67.9 bits (34), Expect = 7e-09 Identities = 67/78 (85%), Gaps = 0/78 (0%)) with the RT-PCR amplified cDNA fragment of rat brain adenosine A_{2a} receptor.

3.3 Multiple Sequence Alignment of cDNA Derived Amino Acid Sequence

In the application to over 700 aligned GPCR sequences from classes A (rhodopsin-like), B (secretin-like) and C (metabotropicglutamate-like), an enhanced evolutionary trace method using Monte-Carlo techniques [39] suggested a potential functional-site on the lipid exposed faces of TM5 and TM6 is common to each family or subfamily of receptors [40] and therefore may be engaged in dimerization interface of GPCRs having specific detectable patterns/motifs. To identify such pattern/motif, single code amino acid sequence (gi|70727927|gb|AAZ07991.1|) of the submitted cDNA sequence of *Rattus norvegicus* (accession no. DQ098650) was retrieved from NCBI protein database. The rat A_{2a} receptor sequence (accession no. DQ098650) was input as a query in PSI-BLAST with inclusion threshold 0.005 (Calculation matrix BLOSUM 62 and E-value 0-1) to find the occurrence of specified pattern in the sequence. Sequences showing identities more than 80% were selected for multiple sequence alignment (Clustal-X) [41]. The results showed that rat brain adenosine A_{2a} receptor protein of 42 residues (gi|70727927|gb|AAZ07991.1|) aligned in TM 5 domain region of AA2AR_RAT (P30543) and exhibited 85.17% homology (36 residues) with TM 5 domain region of AA2AR_HUMAN (P29274) (Fig. 3).

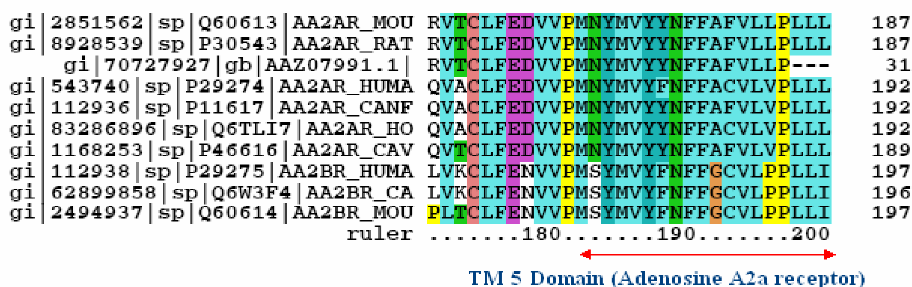

Fig. 3. Homology alignments cDNA sequence of adenosine A_{2a} receptor *Rattus norvegicus* (gi|70727927|gb|AAZ07991.1|) and known mammalian A_{2a} receptor sequences lie in TM5 domain

Previously, it has been reported that TM helix dimerization motifs GxxxG, AxxxA, SxxSSxxT, polar clamps, serine zipper, leucine zipper do not appear in TM5. However, statistical analysis of amino acid patterns in TM helices revealed that PM4 pair (PxxxM) was the most overpresented doublet pattern from any combination of PxxxY doublet pattern suggesting its role in the adenosine A_{2a} receptor dimerization [24].

AA2A_HUMAN (166) CLFEDVV**PMNYM**VYFNFFAC (185)
AA2A_CAVPO (163) CLFEDVV**PMNYM**VYYNFFAF (182)
AA2A_MOUSE (161) CLFEDVV**PMNYM**VYYNFFAF (180)
AA2A_RAT (161) CLFEDVV**PMNYM**VYYNFFAF (180)
AA2A_CANFA (166) CLFEDVV**PMNYM**VYYNFFAF (185)
Q4F987_RAT (8) CLFEDVV**PMNYM**VYYNFFAF (27)
AA2B_CHICK (171) CLFENVV**TMSYM**VYFNFFGC (190)
AA2B_MOUSE (171) CLFENVV**PMSYM**VYFNFFGC (190)
AA2B_RAT (171) CLFENVV**PMSYM**VYFNFFGC (190)
AA2B_HUMAN (171) CLFENVV**PMSYM**VYFNFFGC (190)

Fig. 4. UniProtKB/Swiss-Prot Hits for USERPAT1 {PMNYMV} motif on all (release 51.0), UniProtKB/TrEMBL (release 34.0), PDB (2-Nov-2006) databases sequences

The PROSITE search was carried using PMNYMV residues present in the submitted sequence to examine the occurrence of this pattern in known amino acid sequences of mammalian adenosine receptor. We found that PMNYM motif was conserved in the TM 5 region of all mammalians A_{2a}R and PMSYM motif was present in TM 5 region of all mammalians A_{2b}R. From the detailed domain sequence descriptions employing UniProtKB/Swiss-Prot (Fig. 4 www.expasy.org), we found that 42-residue sequence contains only one PM4 pattern (PMNYM) that lies in the TM 5 domain, the analysis revealed a significant difference in the distribution of conserved TM 5 domain motif

at subtype level of adenosine receptors. In adenosine A_{2a} receptors, the motif PMNYM is highly specific and conserved, however, in adenosine A_{2b} receptor asparagine (N) residue is replaced by serine (S) generating the motif PMSYM [42, 43] thus differentiating the two isoforms of receptors functionally. Finally, we interpret in the context of transmembrane dimerization motifs that conserved motif (PxxxM) may play a role in the dimerization of adenosine receptors .The motif PMNYM of A_{2a}R and PMSYM of A_{2b}R may be involved in TM assembly of the two isoforms of the receptors respectively. The information may provide an insight into the molecular mechanism of receptor-ligand interaction leading to design of tailored compounds.

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