FEBS Letters 351 (1994) 109–113

FEBS 14449

Molecular cloning of the human type VIII adenylyl cyclase

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Received 18 July 1994

Abstract

A cDNA coding for a human type VIII adenylyl cyclase has been isolated from human newborn brain-stem tissue. This cDNA is 6,005 bp long and encodes for a protein of 1251 amino acids, exhibiting the two sets of six transmembrane spanning regions and the hydrophobicity profile typical of other mammalian adenylyl cyclases. Comparison with the rat form shows that they share 97% identity in amino acids. Type VIII adenylyl cyclase is unique in that it has both a long carboxy terminal and a long amino terminal tail. This is the first report on a complete cDNA clone coding for a human adenylyl cyclase. The distribution and regulation of this particular adenylyl cyclase suggest that it may be involved in learning, in memory and in drug dependence.

Key words: Adenylyl cyclase; Human brain; cDNA cloning; Amino acid sequence

1. Introduction

Cyclic AMP is a second messenger for a variety of hormones and neurotransmittors acting via trimeric G protein-coupled receptors. It is synthesized by adenylyl cyclases (AC) which were initially classified as either calmodulin-sensitive or -insensitive enzymes. The extreme diversity of the regulation of the adenylyl cyclase activity was thought to be related to the combinatorial association of a large number of receptors with the isotypes of α, β and γ subunits of the G proteins. In the last few years, it has become clear that the multiplicity of adenylyl cyclase isoforms contributes to the diversity and specificity of this signal transduction system. Indeed, since the purification and cloning of the first AC from bovine brain [1], full-length cDNAs have been reported for seven different types of AC in different tissues and different species [2]. Each of these different cyclases displays specific patterns of sensitivity to the α, β and γ subunits of the G proteins, and to Calcium/Calmodulin (Ca^{2+}/Cam). Until recently, no full-length cDNA for AC had been isolated from human libraries. Only partial cDNA sequences have been obtained for the human forms of AC: in our laboratory, we have isolated, from human brain, partial cDNAs encoding two types of AC, now refered as type II and type VIII, respectively [3,4]; a partial cDNA for type I has been isolated from human fetal brain [5]. In the human genome, all the known adenylyl cyclases are localized on different chromosomes [4–6] and thus their expression might be independently regulated. As part of our effort to study the role of the different adenylyl cyclases in human physiology and disease, we have cloned and completely sequenced a full-length cDNA corresponding to human type VIII adenylyl cyclase.

2. Materials and methods

2.1. cDNA cloning and screening procedure

Total RNA was prepared from newborn brain-stem tissue according to Chomczynski and Sacchi [7]. Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose (Pharmacia) chromatography according to Aviv and Leder [8]. The cDNA library was produced by using the pTZ18R vector (Gibco-BRL) as previouly described [9]. Briefly, the pTZ18R plasmid was linearized using *Pst*I, and (dC)-tailed using terminal transferase. Tailed molecules were digested with *Bam*HI and the small fragment, generated by *Bam*HI cleavage, was removed by spin dialysis through Sephacryl S-400 (Pharmacia). cDNA was produced from 1 μg of poly(A)$^+$ RNA using synthetic oligo(dT) as primer. Residual RNA was removed by alkaline hydrolysis. The cDNA was (dG)-tailed and then selected in size above 3 kb in a low-melting point agarose gel. Finally the 5′ terminal end of the cDNA was converted into *Bam*HI 'sticky-end' by addition of a *Bam*HI adaptor. Subsequently, the single-stranded cDNA was joined to the vector by ligation to produce circular molecules, repair of the single-stranded region was accomplished by using the (dC) tail of the vector as a primer and T4 DNA polymerase (Gibco-BRL).

Approximately $2 \cdot 10^6$ clones were screened using, as probes, radioactive oligonucleotides complementary to the previously described sequence [3]. The probes were radiolabelled using [α-^{32}P]dCTP and terminal deoxynucleotidyltransferase according to the standard method [10]. Prehybridization and hybridization were performed in 6 × SSC, 3 × Denhardt's and 0.5% SDS for 2 h at 42°C and filters were washed 3 times with 6 × SSC, 0.1% SDS at 50°C for 20 min. Two positive overlapping clones were obtained, only the longer one (6,005 bp) was completely sequenced.

2.2. DNA sequencing and sequence analysis

Fragments of the cDNA have been subcloned in the pTZ18R vector. Both strands were sequenced by the dideoxynucleotide method, from a set of subclones and using different synthetic oligonucleotides [11]. A computer program based on the method described by Kanehisa [12], and using the matrix of Dayhoff [13] was used to define regions of homologies between the amino acid sequence of human type VIII AC and the sequences of all the known mammalian AC species. Alignment

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SSDI 0014-5793(94)00836-1

was performed using the Clustal program [14] Sequence analysis was performed using the programs of the genetic computer Group Package (UWGCG). A search for homologous sequences was performed using the SwissPro and Gene EMBL databases. The sequence has been submitted to EMBL databank under the Accession Number Z35309.

3. Results and discussion

A 2,183 bp cDNA fragment encoding the C-terminal 683 amino acid sequence of the human type VIII adenylyl cyclase has previously been identified from a human brain (frontal lobe) cDNA library, and was initially referred to as type V [3]. Using oligonucleotides complementary to the 5′ end of this sequence and a modified RACE protocol [15], several overlapping clones were obtained which allowed us to determine an incomplete 2,988 bp cDNA sequence. Different oligonucleotides from this sequence have been used to screen a new cDNA library from human newborn brain-stem leading to the identification of one clone of 6,005 bp. Partial sequencing and restriction analysis confirmed the presence of the previously identified 3′ end sequence in this clone. The first putative initiation codon, in a context of a reasonable Kozak consensus sequence [16], is preceded by a 2,094 bp of non-coding sequence including stop codons in all three phases. In particular we have identified a stop codon (UAG) 60 bp upstream of this AUG in the same frame. This region is highly GC-rich. The open reading frame encodes a protein of 1,251 amino acids (Fig. 1) with a deduced molecular weight of 140 kDa. The hydrophobicity profile of the type VIII adenylyl cyclase is similar to that of the other membrane bound adenylyl cyclases with the 12 predicted transmembrane spanning regions [17]. Three putative N-linked glycosylation sites exist between membrane spanning regions 9 and 10 at amino acid positions 817 and 821, and between membrane spanning regions 11 and 12 at amino acid position 887, as found for most of the adenylyl cyclases. A potential protein kinase A phosphorylation site can also be detected in the second portion of the first cytoplasmic loop, as also described for adenylyl cyclases types II, III,V and VI [17–21].

Recently, Cali et al. [22] published the sequence of a type VIII AC from rat brain; a 88% identity in nucleotide sequence and 97% identity in amino acid sequence exists between these two forms (Fig. 1). The major difference between these two cyclases resides in the absence of three serine residues in the NH_2 terminal part of the rat molecule. It is noteworthy that the carboxy-terminal cytoplasmic domains of human and rat type VIII have more than 90% identity in amino acid sequence. The same region of human and bovine type I is less conserved across species [5]. No significant homology was found between type I and type VIII in this part of the molecule.

Type VIII adenylyl cyclase is unique among the cyclases in having both a long NH_2 terminal tail as in

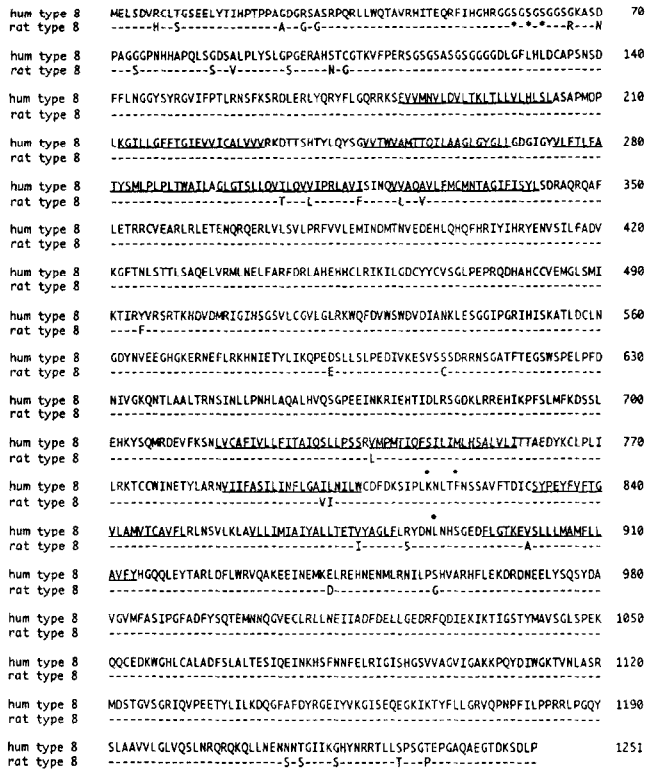

Fig. 1. Comparison of deduced amino acid sequences of human and rat type VIII adenylyl cyclases. Alignment of human and rat type VIII adenylyl cyclases amino acid sequences. Residues identical in both sequences are indicated with (–). (*) Represents the Serine gaps in the rat sequence. (●) Indicates the positions of the putative N-glycosylation sites. Predicted trans membrane regions are indicated as underligned sequences.

type V and VI (type VIII: 182 aa; type V: 164 aa: type VI: 150–160 aa; type I: 63 aa; type II: 44 aa; type III: 77 aa; and type IV: 28 aa), and a long COOH terminal tail as in type I (type VIII: 80 aa; type I: 76 aa; type II: 13 aa; type III: 23 aa; type IV: 14 aa; type V: 5 aa; and type VI: 4 aa). These N- and C-terminal regions may support specific functions for the different cyclases. By expressing rat type VIII in 293 cells, Cali et al. [22] have demonstrated that this adenylyl cyclase is sensitive to Calcium/Calmodulin, a property that is shared with type I and type III AC. Using point mutagenesis, it has been shown that amino acids within the sequence 495–522 of type I adenylyl cyclase contribute to the CaM binding domain, but no similar sequence is found in human type VIII. Sequences that are involved in calmodulin binding usually contain a mixture of cationic and hydrophobic amino acids that can form an amphiphilic alpha helix [23], such structures may exist in different regions of the type VIII molecule.

Using computational matching programs, the amino acid sequences of all the known 16 mammalian adenylyl cyclases have been aligned. Sequence comparison had allowed one to define 8 different types; within each type, adenylyl cyclases from different species shared 88–97%

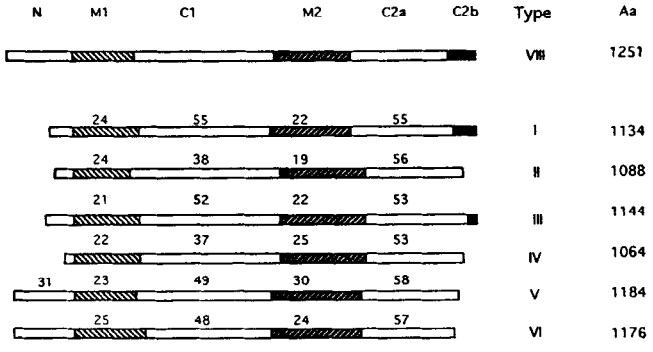

Fig. 2. Comparison of the amino acid sequences of the different adenylyl cyclases with that of human type VIII adenylyl cyclase. The relative positions of the different domains has been determined from the alignment of all the known AC and the positions of the sequences which are predicted to form membrane associated helices. A computer program based on the method described by Kanehisa [12], and using the matrix of Dayhoff [13] was used to define regions of homologies between the amino acid sequence of human type VIII AC and the sequences of all the known mammalian AC. Alignment was performed using the Clustal program [14]. Since within one AC type the identity is at least of 90%, one member of each type is reported for the comparison (Bovine type I [1], Rat type II [19], Rat type III [18], Rat type IV [35], Canine type V [20] and rat type VI [21]. The number reported above each domain represent the degree of identity of this domain with the corresponding domain of human type VIII AC. Data about type VII are too sketchy to be included in the present scheme.

identity in amino acids, whereas only 30 to 35% identity is detected between cyclases from different types; these differences reside mostly in the trans-membrane spans. Alignment of the amino acid sequences and comparison of the percentage of amino acid identity clearly reveals the presence of 5 major domains, as already proposed from the hydrophobicity profiles: the NH_2 terminus (N), the two trans-membrane regions (M_1 and M_2) and the two cytoplasmic catalytic loops (C_1 and C_2). Fig. 2 shows the comparison between these different major domains of the different types of adenylyl cyclases with the corresponding domains of the human type VIII. As mentioned above, the carboxy terminal part of the C_2 domain (C_2b) is only present in type I and type VIII adenylyl cyclases, and to a lesser extent in type III. Type I AC has been described as being neural specific [24], and type VIII AC appears to be essentially expressed in the brain [22,25]. There is no homology between type I and type VIII AC in the carboxy terminal part of the molecule. In the NH2 terminal domain (N), a low level of identity (31%) is found between type VIII and type V, and no homology exists with type VI; this region is highly type specific. The amino terminal part of the C2 domain (C2a) is the most conserved part of the enzyme between the different types (more than 50% amino acid identity). The second most conserved region is the cytoplasmic C_1 domain; a high degree of homology exists between C_1 and C_2 in all the types of adenylyl cyclases and both domains are required for the catalytic activity [26,27]. In this region, type VIII has a lower homology with type II and type IV than with the other adenylyl cyclases. The trans-membrane domains M_1 and M_2 appear to be type specific, since they differ from one cyclase to another (approximately 20% identity); however some positions are conserved in the different types (Fig. 3): Tyr at position 174, and Leu, Pro at position 177–178 of human type VIII both at the end of the 4th putative trans-membrane span, Gly at the end of the 6th span, and Lys at the end of the 11th span; all these conserved positions are on the cytoplasmic side of the molecule, and thus might be involved either in the catalytic site or in the interaction with the Gα subunit. Moreover, between the ninth and the tenth span, all the cyclases have one or two putative N-glycosylation site(s). Fig. 3 shows the conserved positions between the different adenylyl cyclases. It is noteworthy that in all the cases, a tyrosine is present 9 amino acids before the M1 domain, another tyrosine is also present 3 amino acids before the M2 domain. Another striking observation made from sequence com-

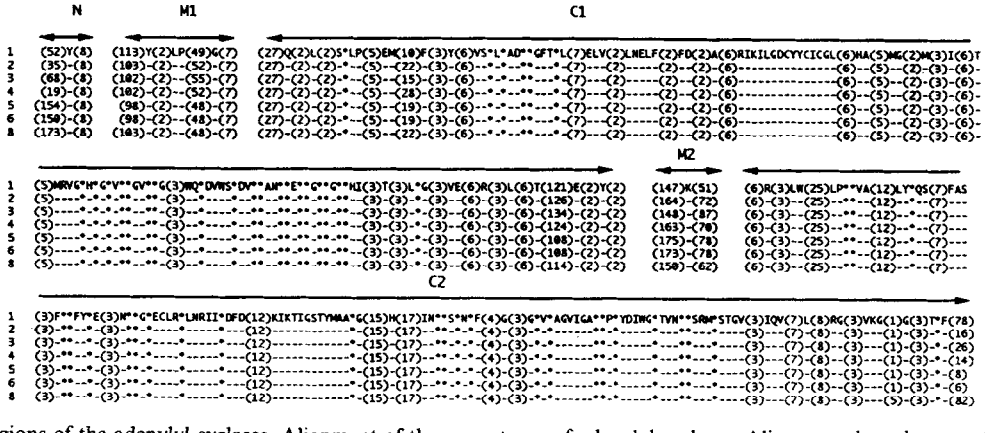

Fig. 3. Conserved regions of the adenylyl cyclases. Alignment of the seven types of adenylyl cyclases. Alignments have been performed as decribed under legend to Fig. 2. 1,2,3,4,5,6, and 8 are the numbers of the different types of adenylyl cyclases. Residues identical in all the AC are indicated by: (−) Numbers in parentheses correspond to the number of amino acids in the less conserved regions. Most conserved modifications appeared as (*). The nature of these amino acids are not indicated.

parison was the fact that conserved amino acids or stretches of amino acids are separated by spaces of conserved length (Fig. 3). All the conserved positions are likely to confer to the adenylyl cyclases both their structure and their catalytic activity.

By Northern blot analysis we found two types of mRNA of 10.5 and 5.5 kb in human brain [3]. In rat brain, using the type VIII oligonucleotide specific probe, described by Cali et al. [22] for in situ hybridization, we found the two major forms of appoximately 4.4 and 5.5 kb as described [22], but also a faint band at 7.5 kb. In human brain, we did not find the 4.4 kb form . All those results suggest the presence of several forms of AC type VIII . Indeed, only one gene for type VIII AC has been detected on human chromosomes (8q24); It is thus very likely that the different forms are produced either via an alternative splicing or use of alternative promotors or of different polyadenylation signals. Similar hypotheses have been proposed to account for the different forms of type V AC in canine heart mRNAs [20]. In addition, by screening a λGt10 cDNA library from human testis, we isolated a partial 2 kb cDNA sequence which is identical to the 3′ end of the human brain type VIII, but contains a 90 bp deletion localized in the second transmembrane domain leading to the loss of the region between the membrane spans 9 and 10, and of two potential N-glycosylation sites (not shown). The complete cDNA sequence of this AC has not yet been obtained and we do not know if other deletions are present in this AC form in testis.

Because of the structural similarity of type I and type VIII AC, it was of interest to compare their relative distribution in various regions of the brain. By in situ hybridization, type VIII adenylyl cyclase has been found to be expressed in unique structures of the brain, particularly in the pyramidal and granular cells of the hippocampus, but also in several nuclei of the brain stem such as the pontine nuclei, the facial nucleus, lateral reticular nucleus and the locus coeruleus [22, 25]. This distribution is compatible with the distribution of the Ca^{2+}/Cam sensitive adenylyl cyclase activity and the distribution of the Cam mRNA [25]. Both type I and type VIII are expressed in several regions of the brain which are associated with learning and memory, but with distinct relative levels of expression [22,25,28,29]. Cali et al. thus proposed that both type VIII and type I are required for the development of long-term potentiation in the hippocampus [22]. The important role of the AC type VIII in brain function was strongly supported by the observations made on its regulation by opioids. Indeed acute opioid administration decreases the AC activity both in cell culture and in brain from treated animals, by acting on G-protein coupled receptors [30–33]. It has been proposed that during chronic exposure to morphine, neurons could dramatically increase their AC activity to overcome the constant inhibition of the enzyme. In a recent paper, we provide direct experimental evidence that chronic morphine treatment in mice induces a selective increase in type VIII AC mRNA transcripts in the locus coeruleus, the amygdala and the thalamus, all structures which have been shown to be critically involved in morphine abstinence [34].

At present, eight different types of AC have been reported [2]. For seven of them, the chromosomal localization has been determined [4–6]; all of them are localized on different human chromosomes and might be, at least in part, independently regulated at the gene level. The gene for type VIII adenylyl cyclase is located on chromosome 8 at the q24 band; two important genes are located in the proximity of this gene: the c-*myc* oncogene and the thyroglobulin gene. In Burkitt's lymphoma, c-*myc* is translocated on a different chromosome near the immunoglobulin gene cluster, thus modifying its expression. It would therefore be interesting to study the expression of type VIII AC in Burkitt's lymphoma. This should now be possible since the entire sequence of the cDNA is available. To date, no pathology has been directly assigned to a genetic change in the expression of any type of adenylyl cyclase. Most of the known alterations in the production of cyclic AMP originate from other components of the cyclase system, hormonal recptors or G proteins. Again, the availability of entire sequence of human adenylyl cyclase should help the search for alterations in the structure of AC associated with human diseases.

Acknowledgements: We are grateful to Robert Barouki for critical reading of the manuscript, and to Lydie Rosario for expert secretarial assistance. This work was supported by the 'Institut National de la Santé et de la Recherche Médicale', the 'Université Paris-Val-de-Marne', the Fondation de France, the 'Caisse Nationale d'Assurance Maladie' and the 'Ministère de la Recherche et de la Technologie'. Alena Danisova, on leave from the Slovak Academy of Sciences (Bratislava), was a recipient of a fellowship from the Ministère de la Recherche et de la Technologie. Isao Matsuoka, on leave from the Fukushima Medical School (Japan), was a recipient of a fellowship from Fondation de la Recherche Médicale, and Vadim Iourgenko, on leave from the Ukrainian Academy of Sciences (Lviv), was a recipient of a fellowship from Ministère de la Recherche et de la Technologie.

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