MOLECULAR CLONING OF THREE ADENYLYL CYCLASE ISOFORMS FROM THE SHARK SALT GLAND

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Secretagogues stimulate chloride secretion in the rectal gland via two second messenger systems, cyclic AMP (cAMP) and cyclic GMP (cGMP). Whereas C-type natriuretic peptide (CNP) activates rectal gland secretion by increasing intracellular cGMP (Aller et al., *Am J. Physiol.* 276: C442-9, 1999), other secretagogues including adenosine, vasoactive intestinal peptide (VIP), growth hormone releasing hormone (GHRH) and pituitary adenylate cyclase activating peptide (PACAP) act via seven transmembrane G protein coupled receptors (7TM-GPCRs), which activate adenylyl cyclase (AC) (Forrest, *Kidney Int.* 49:1557-62, 1996). AC is a crucial effector enzyme responsible for converting ATP into cAMP.

Ten AC isoforms have been identified in mammals (AC1-AC10). These proteins can be subgrouped by their regulatory features. All share common regulatory properties: activation by the α subunit of the heterotrimeric G-Protein (Gsα), stimulation by the diterpene forskolin (except AC9) and inhibition by a class of adenosine analogs known as P-site inhibitors. The isoforms of mammalian ACs share a primary structure consisting of a variable length amino-terminal cytoplasmic tail (N), two transmembrane regions, M1 and M2, and two cytoplasmic regions, C1 and C2 (Figure 1). The membrane regions each contain six transmembrane-spanning helices (6TM). The function of M1 and M2, aside from membrane localization, is unknown, despite their topological analogy to ABC-transporters. The C1 and C2 regions are further subdivided into C1a and C1b; and C2a and C2b. The C1a and C2a regions are well conserved, homologous to each other, and contain all of the catalytic apparatus. The C1b region contains several regulatory sites. The C2b region is short and contains regulatory sites in some ACs.

Our aim was to determine which ACs are expressed in the rectal gland in order to identify the isoform(s) which play a role in chloride secretion in this organ. Because each subtype is regulated differently, information about which subtype(s) is (are) expressed in the gland would allow us to draw conclusions concerning the regulatory functions of AC in the modulation of chloride secretion in the gland.

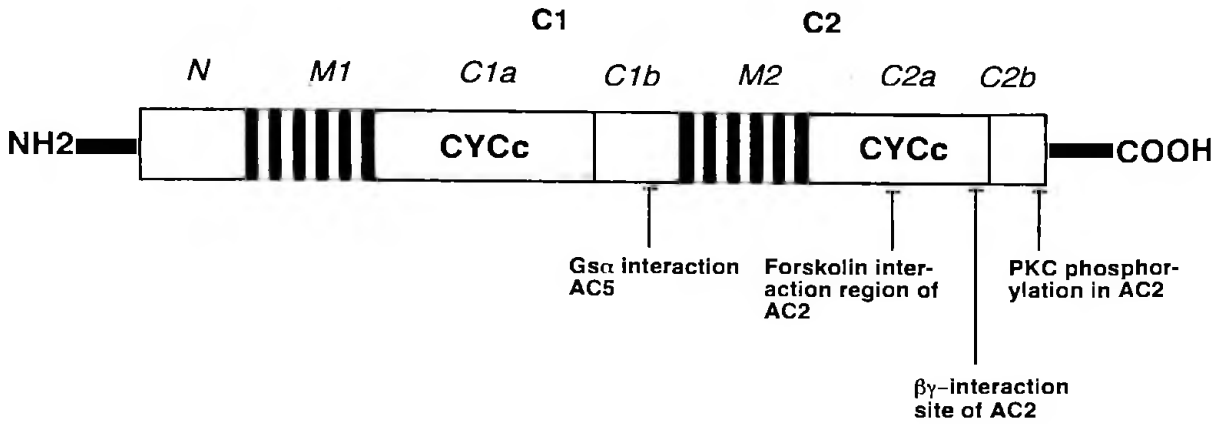


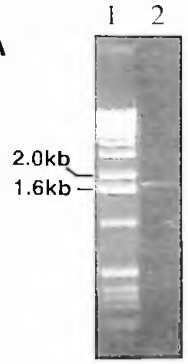
Figure 1. Structure of mammalian adenylyl cyclases. (see text for explanation)

We used a CsCl method to prepare total RNA from a fresh rectal gland, which was perfused for 2 minutes with shark Ringer's solution prior to the extraction. Subsequently reverse transcription was performed using the cDNA Cycle Kit (Invitrogen, Carlsbad, California). Degenerate primers were designed, using an alignment of various AC isoforms from eight species found in the GenBank database. 5'-GGNGAYTGYTAYTAYTGYGT-3' was used as a forward and 5'-CCDATNGTYTTDATYTTYTC-3' as a reverse primer. PCR using these primers was performed in an Eppendorf Master Cycler with the following parameters using the hot start technique and Expand High Fidelity (Boehringer Mannheim): 95°C 4min; 35 cycles (95° 30s; 47°C 30s; 68°C 90s). This degenerate PCR resulted in a single 1.8kb band (see Figure 2), which was then cloned into the pCR2.1 vector (TA-Cloning, Invitrogen).

To identify potential heterogenous inserts, 20 clones were subjected to a restriction enzyme digest using the multi-cutting enzymes RsaI and HhaI. Three different restriction enzyme patterns were identified and one representative clone for each (A1-6, A1-14, A1-24 was sequenced. The most abundant was the A1-14 restriction pattern with a ratio of 13:5:1 for A1-14, A1-24 and A1-6 respectively. A1-6 had a total length of 1835 bp, coding for 611 amino acids, A1-14 had 1817 bp coding for 605 amino acids and A1-24 had 1778 bp coding for 592 amino acids. All three sequences had highest homologies to AC when examined by a BLAST search (GenBank). To obtain complete sequence, nested RACE-PCR was performed using the Marathon-RACE kit (Clontech, Palo Alto, CA). For each degenerate PCR product, two touchdown RACE PCRs were performed using a gene specific primer (GSP) for each the 5' and 3' end of each sequence and the clontech adapter primer (AP1). Then 1µl from each reaction was used as a template for the second reaction using a nested gene specific primer (NGSP) and the nested adapter primer (NAP). The first reaction of each PCR did not result in any visible PCR product. The results of the nested PCR for the 5' and 3' end are summarized in Figure 2, Panel

B. Sequences were cloned and sequenced up to the poly-A-tail at the MDIBL Marine DNA Sequencing facility. Lasergene software (DNASTAR,Madison, WI) was used to edit and assemble the sequences and to identify the open reading frames (ORF).

A



B

Clone ID	5'Race product	3'Race product
AC1-6	1.6kb	2kb
	1.2kb	
	0.9kb	
Ac1-14	1.6kb	2kb
AC1-24	1.4kb	2.4kb

Figure 2: Panel A. Lane 1: markers; lane 2: PCR product of adenylyl cyclases from shark rectal gland cDNA. Panel B. 5'- and 3'-Race products obtained from the nested RACE PCR.

The consensus sequence of clone A1-6 and the 5'-and 3'-Race products yielded 4088 nucleotides. We identified an 3.27 kb ORF encoding 1089 amino acids. The hydrophilicity plot predicted a protein with two 6 TM domains, each followed by a large hydrophilic region. The protein has highest similarity (61%) to rat type 2 AC, which is the only full-length AC type 2 sequence in GenBank. Highest homology is observed in the C1A (80%) and C2A (83%) domains, which are crucial in catalytic function. These regions are highly conserved in all known adenylyl as well as guanylyl cyclases (Krupinski et al., *Adv. Sec. Mess. Phos. Res*. 32:53-79, 1998). M2 (42%) and C2b (51%) domains showed lowest similarity between the rat AC2 and the shark AC2-like AC.

AC 2 can be classified by its regulatory features into a group with AC4 and AC7. These cyclases have been shown to be stimulated by Gsα and superstimulated by Giβγ (Gilman et al., *Science* 254, 1500-03, 1991). Unlike all other ACs, AC2 is extensively stimulated by activation of PKC (Yoshimura et al., *J Biol Chem.* 268: 4604-7, 1993) and the PKC phosphorylation site has been localized to Thr 1057 of the of rat AC2 C2b region (Levin et al., J Biol Chem,;270:7573-9, 1995). However in the shark AC2-like AC, this threonine is replaced by a histidine. Rat AC2 is exclusively expressed in rat brain and lung as demonstrated by northern blot (Feinstein et al, *PNAS*:1991;88:10173-77).

Consensus sequence of clone A1-14 and the 5'-and 3'-RACE products resulted in a 3992 bp nucleotide sequence. We identified an ORF of 3.486 kb encoding for 1165 amino acids. This

protein has highest homology to rabbit AC 5 (71%). Maximum similarity was again observed in C1A (95%) and C2 (91%) domains, whereas identity in the regions C1B (65%) and M1, M2 (63, 63%) where substantially lower. The N-terminus showed only a 28% homology between shark and rabbit. The rabbit AC5 has an additional 76 amino acids at the N-terminus.

AC5 can be subgrouped into the family of calcium-inhibitable ACs (AC5/6). AC5/6 are inhibited by micromolar amounts of intracellular calcium elevated through capacitive calcium entry (Chiono et al. *J Biol Chem*, 270:1140-55, 1995). Furthermore AC5/6 is stimulated by Gsα and inhibited by Giα and Gzα. Recently NO was found to have a direct inhibitory effect on AC6, most likely due to S-nitrosylation of a cysteine residue (McVey et al., J Bio Chem;274:18887-18892, 1999). AC5/6 are most abundantly expressed in heart and to a lesser degree in brain, kidney, liver and testes. In microdissected rat renal glomeruli and nephron segments, AC6 mRNA (detected by RT-PCR) was present all along the nephron. Type 5 mRNA was restricted to the glomerulus and the initial portions of the collecting duct (Chabardès et al., *J Biol Chem*, 271:19264-71, 1996).

By sequencing several clones of the shark AC5-like-AC, we have also identified a possible splice variant of this protein. It is 100% homologous to sharkAC5-like-AC but lacks amino acids 282-332. Splice variants for AC have been reported for AC8, AC5 and AC4 (Krupinski et al., *Adv. Sec. Mess. Phos. Res.* 32:53-79, 1998).

AC1-24 and its 3'-and 5'-RACE products resulted in a consensus sequence of 4562 nucleotides. No start codon could be identified in the 5' region, most likely due to sequencing errors because of GC rich sequence. The degenerate product and the 3'Race product could be translated in a 952 amino acid protein fragment which showed highest similarity (59%) to bovine AC7.

In summary, we have cloned two full length adenylyl cyclases from the shark rectal gland with highest homology to mammalian AC2 and AC5 and one 952 amino acid fragment of another shark adenylyl cyclase most analogous to mammalian AC-7. The level of expression and localization of these proteins to cell types and membrane domains in the rectal gland will be assessed in future experiments. However, functional data from rectal gland perfusions (stimulation of cAMP by Gsα coupled receptors and inhibition by Giα coupled receptors such as somatostatin) suggests that the predominant AC subtype is likely to be a member of the Ca-inhibitable subgroup of adenylyl cyclases (AC5 and 6).

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