Cloning and expression of a bovine adenylyl cyclase type VII specific to the retinal pigment epithelium

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Abstract **A cDNA of a type 7 adenylyl cyclase isoform was cloned from a bovine retinal pigment epithelium cDNA library using oligonucleotides developed to conserved regions common to mammalian adenylyl cyclases. A 6.7 kb mRNA of very high abundance was uniquely present on Northern blots containing mRNA or total RNA from the pigment epithelium. This transcript was undetectable in all other tissues examined. The cDNA encoded a protein of 1,097 amino acids and exhibited the known doublet of 6 transmembrane-spanning regions in a hydrophobicity plot. The novel member of the type 7 adenylyl cyclase isoform was expressed in COS-1 cells. It was stimulated 10- and 20-fold by 10 μM GTPγS and 100 μM forskolin, respectively. The high expression rate exclusively in the retinal pigment epithelium suggests that this adenylyl cyclase isoform is involved in processes specific to this functionally exceedingly important subretinal cell layer.**

Key words: Adenylyl cyclase; Retina; Retinal pigment epithelium; cDNA cloning; Expression

1. Introduction

To dat 9 mammalian AC isoforms (types 1–8, and 10) have been cloned and sequenced ([1–13]; for a review see [14]) All isozymes show the same pattern in their suspected membrane topology, i.e. two sets of 6 membrane spanning regions are connected via a large cytosolic loop. All isoforms are stimulated by the diterpene forskolin and GTP. Based on comparisons of amino acid similarities, types 2, 4 and 7 represent one subfamily of related isozymes, types 5 and 6 appear to define a second subfamily whereas the other isoforms, 1, 3, 8 and 10, seem to stand alone. An important difference between various AC isozymes is that they are distinctly regulated by the α or βγ subunits of the heteromeric GTP-binding proteins (G-proteins), by Ca^{2+}/calmodulin and by phosphorylation [15–18]. Although these regulatory studies have not sufficiently covered all isoforms to allow for an assignment into certain subgroups based on regulation it is becoming increasingly clear that a classification scheme along these lines will probably emerge. The functional diversity of this growing family of isozymes is evident from the distinct expression of particular isoforms in different cell types and tissues [15,19].

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The nucleotide sequence reported here has been submitted to the EMBL/GenBank data base under the Accession Number Z49806.

Abbreviations: AC, adenylyl cyclase; PCR, polymerase chain reaction; RPE, retinal pigment epithelium; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate).

We attempted to identify the AC isoforms which are expressed in the bovine retina using a PCR-based strategy and retinal cDNA libraries. In addition to type 1, 5 and 8 AC clones we obtained a 1.5 kb fragment of an AC isoform which was not previously reported. It turned out that this PCR product was the result of a cDNA carried over from the RPE during library preparation. The corresponding AC full length clone was expressed with an extraordinary tissue specificity and in high amounts in the RPE and not in the morphologically adjacent retina.

2. Material and methods

2.1. cDNA cloning

Oligonucleotides used were: J399, GGGAYTGYTAYTAYTG; J400, TTNCCCCADATRTCRTA; J401, TGGCARTWYGAYGTNTGG; J402, TCRAARTCNGCDATDATYTC; R9, GARAAGATYAARACCATHGG; R10, ACIGTRTTICCCCARATRTC; K1, GCCACCATGCTCCCCAGGCCGTGG; K2, GCCACCATGCCAGCCAAGGGGCGC; 2U, AGGCAGGCCCCAGATGAG; AC7S, CCTTCAGCYAKGGGGACCCCTCCAG; AC7A, GCAGYAGYGTGTACACCACGAAGAC; (abbreviations according to the IUPAC code).

Total RNA from 30 bovine retinas or 360 bovine pigment epithelia was isolated by isopycnic centrifugation in Cs^+ trifluoroacetate after tissue homogenization in 5.5 M guanidinium isothiocyanate [20]. RNA from KG-1 leukemia cells and different rat tissues was prepared with the RNEasy kit (Qiagen). Poly(A)$^+$RNA was enriched by chromatography on oligo(dT) cellulose.

Synthesis of cDNA was accomplished with either the superscript-cDNA-synthesis-kit (Gibco-BRL) or λ-ZAPII-cDNA-Synthesis kit (Stratagene). cDNAs were size fractionated (1–12 kb) and adapter-ligated prior to generation of oligo-(dT) primed libraries in either λ-ZAPII/*Eco*RI/CIAP (retina) or λ-ZAP-Express/*Eco*RI/*Xho*I/CIAP (RPE).

Polymerase chain reactions were carried out in 50 μl containing 10 ng sscDNA, 50 pmol of each primer, 200 μM of each dNTP and 1 U of Taq-DNA-Polymerase (Boehringer, Mannheim). Standard screening and cloning procedures were used as described in [20]. Hybridizations were for 16 h at 42°C in 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM Na-citrate, pH 7.0), 0.1% SDS, 50% formamide, 100 μg/ml denatured herring sperm DNA, 0.5% ficoll 400, 0.5% polyvinylpyrrolidone 100, 0.5% BSA. cDNA libraries were screened with a 996 bp *Nco*I/*Xba*I fragment prepared from a 1.5 kb PCR product which was generated with J399/J401 as primers and a retinal cDNA library as a template and subsequently J400/J402 as nested primers and 5 μl of the foregoing reaction as a template. The probe was labelled by random-priming (DECA*prime*, Ambion). Filters were washed twice for 30 min at 55°C in 1 × SSC, 0.1% SDS. Positive single plaques were excised with *ExAssist-Helper-phage* and clones were obtained in the expression plasmid pBKCMV (Stratagene). After subcloning into pBluescript SK^-. DNA fragments were sequenced by the dideoxy chain termination method [21].

2.2. Northern blot analysis

2–5 μg of poly(A)$^+$RNA or 20 μg total RNA isolated from the retina, the RPE and other tissues were run on a 0.65% agarose/formaldehyde gel, blotted onto nylon membranes and crosslinked by UV irradiation.

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A Mouse-Multiple-Tissue-Northern Blot with 2 μg mRNA from several tissues was purchased from Clontech (# 7762-1). The blots were hybridized for 24 h with the *NcoI/XbaI* fragment (see above, $4.8 \cdot 10^8$ cpm/μg DNA) and washed twice in 1 × SSC, 0.1% SDS for 30 min at 63°C.

2.3. Expression of the AC in COS-1 cells

A 6.2 kb cDNA full length clone in pBKCMV was shortened by sequential digestions with *KpnI*, *PstI* and *EcoRI*. The truncated 4.5 kb clone which contained 240 bp of the 3′-noncoding region and 525 bp 5′ to the first ATG start site, was ligated into the *EcoRI/PstI* site of the pCMV5 expression vector [22]. Since the start ATG triplett was not unambigously identifiable, Kozak sequences were placed in front of the first and second ATG codons, using primers K1 and K2 as sense primers and 2U as antisense primer. The PCR products were blunted, restricted with *ApaI* and ligated into the pCMV5-AC7-construct which had been treated sequentially with *EcoRI*, Klenow fragment and finally digested with ApaI. COS-1 cells were seeded into 75 cm^2 flasks and after 2 d cells were transfected with 5 μg of supercoiled plasmid-DNA by the DEAE-dextran method including treatment with chloroquine and a DMSO shock [23]. After 3 d cells were harvested into 5 ml of phosphate-buffered saline and collected by centrifugation (6,200 × g, 15 min). The cells were suspended in buffer (20 mM Tris-HCl, pH 7.5, 20% glycerol (v/v), 1% thioglycerol, 1 mM EDTA, 1 mM benzamidine, 0.1 mM PMSF) and homogenized by passing through a 23-gauge needle ten times. The samples were stored at −80°C.

2.4. AC assay

Enzyme activity was measured for 10 min at 37°C in buffer containing 50 mM Tris-HCl, pH 7.5, 22% glycerol (v/v), 2 mM Mn^{2+}, 2 mM [^{3}H]cAMP (for determination of yield) and 75 μM [α-^{32}P]ATP. cAMP formed was isolated by chromatography [24]. Protein determination was carried out with the Lowry method.

3. Results

3.1. Retinal adenylyl cyclases

Using the degenerate primer pairs J399/J401 or J400/J402 (internal primers) directed against conserved stretches of known mammalian ACs and a bovine retinal cDNA library as a template we did not amplify the expected distinct 1.8 or 1.5 kb fragments. Using the primer pair J399/401 for the first PCR and J400/J402 as nested primers in a second PCR yielded an expected 1.5 kb band corresponding in size to a potential AC. Indeed, the amino acid sequence of the sequenced fragment was similar to mouse type 7 (76%, [10]) and human type 7 (83%, [25,26]) ACs. A stretch of 996 bp located between *NcoI/XbaI* sites which was most divergent at the DNA level, was used to screen several bovine retinal cDNA libraries. No positive clones were detected. However, using the degenerate AC-specific primers R9/R10 we readily and unequivocally identified the expression of AC types 1, 5 and 8 at a clonal ratio of 1:5:2 in the retina. The failure to detect any clone corresponding to the type 7-like fragment indicated that it either was an extremely rare transcript or an impurity of the retinal cDNA library from an adjacent tissue.

3.2. Type 7 AC in the retinal pigment epithelium

Next we prepared a Northern blot with 5 μg poly(A)$^+$RNA or 20 μg total RNA from the bovine retina, the adjoining RPE and several other tissues and hybridized with the 996 bp DNA fragment (Fig. 1). Unmistakenly, the novel type 7 AC isoform was exclusively expressed in the RPE whereas it was undetectable in all other tissues. Obviously, the transcript was very abundant in the RPE since it required only an overnight exposure of the hybridized blot to see a strong signal at 6.7 kb (Fig. 1). An additional exposure of the blot for another 7 d indicated that this transcript was indeed completely absent in the retina and in the other tissues tested (data not shown). Further, a Mouse-Multiple-mRNA blot (Clontech) with 2 μg of poly(A)$^+$-enriched RNA from kidney, skeletal muscle, liver, lung, spleen, brain and heart was negative (data not shown).

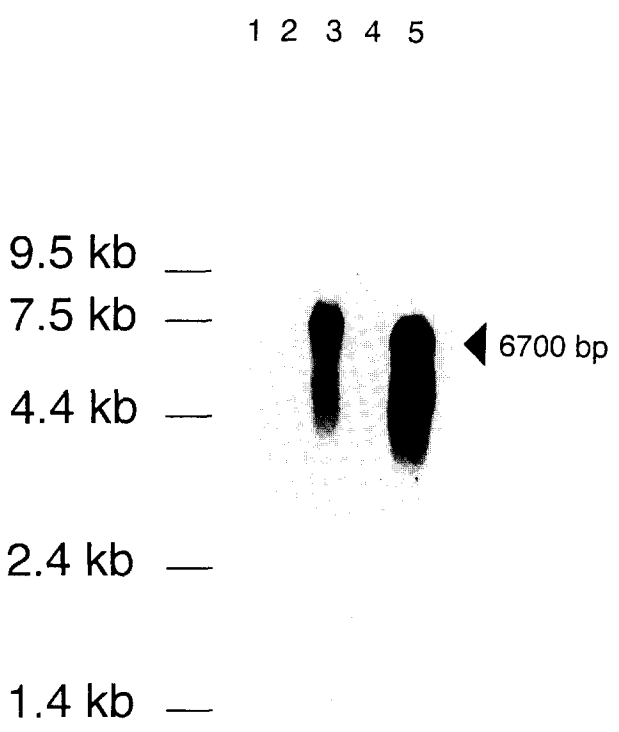

Fig. 1. Northern Blot analysis of poly(A)$^+$RNA (5 μg/lane) or total RNA (20 μg/lane) of several mammalian tissues. The probe was the DNA stretch from basepairs 2125 to 3120. Shown is an autoradiograph after 16 h of exposure. Molecular size RNA markers (Gibco-BRL) are indicated. Individual lanes contained total RNA from: (1) rat brain; (2) bovine retina; (3) bovine RPE; (4) rat liver; and (5) poly(A)$^+$RNA from bovine RPE. In the same blot no signals were detectable with retina poly(A)$^+$RNA, total RNA from KG1-human leukemia cells and the follwing rat tissues: kidney, thymus, heart, lymph nodes, lung, spleen, and testis (data not shown).

Next, we used primers, R9/R10, which were developed to conserved regions common to all mammalian ACs and sscDNA from the RPE as a template in a PCR to identify all AC isoforms expressed in the RPE. After subcloning the PCR products into pBluescript and sequencing we obtained 25 clones of the novel type 7 AC and only one single clone encoding a type 2 AC. The type 2 AC was, however, not detectable in bovine RPE poly(A)$^+$RNA by northern blot analysis using an AC type 2 specific probe (data not shown). This demonstrated that the type 7-like AC isoform is probably by far the most abundant AC expressed in the RPE in vivo. We also examined whether the type 7 AC is expressed in human RPE using a cDNA library established from cultured human RPE (ATCC 77443) as a PCR template and the specific primers AC7S and AC7A. We easily identified the human type 7 AC

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1    MLPRPWGFLRGPLGVPRWRMPAKGRYFLNEGEEGPDQDALYEKYRLTSQH
51   GPLLLMLLLVAIAACTTLIVITFSYGDPSRHRAVLGTAFFTLAMFVLLYA
101  LVYVECLDRRGLRISALLIWGCLVTLGYVLVFDFDSPRKDTLCLWGRCPS
151  SSFVVFVVYTLLPFSMWGAVTAGLVSSISHLLVLAMHQEDFTSPVGLKLL
201  ATAVVFVCGNLTGAFHKIIHMQDASHDLFTYTVKCIQIRRKLRIEKRQQEN
251  LLLSVLPAHISMGMKLAIIERLKERGDRRYLPDNNFHNLYVKRHQNVSIL
301  YADIVGFTRLASDCSPKELVVVLNELFGKFDQIAKANECMRIKILGDCYY
351  CVSGLPVSLPNHARNCVKMGLDMCEAIKQVREATGVDISMRVGIHSGNVL
401  CGVIGLRKWQYDVWSHDVSLANRMEAAGVPGRVHITEATLKHLDKAYEVE
451  DGHGQQRDPYLKEMNIRTYLVIDPRSQQPPQPSQHNSKNKGNATLKMRAS
501  VRMTRYLESWGAARPFAHLNQRESVSSSETLVSHGRRPKAVPLRRHRTPD
551  RSASPKGRSEDDSYDDEMLSAIEGLSSTRPCCSKSDDFSTFGSIFLEKGF
601  EREYRLAPIPRVRYYFACASLVFVCILLIHVLLLYSMKTLGVSFGLVACV
651  LGLVLGLCFADVFLRCCPALGKLRAIAESVETQPLLRVSLAILTIGSLLV
701  IAVVNLPLMPFRDRGLTAGNETGLRAVSGWEMSPCYLLPYYTCSCILAFI
751  ACSVFLRMSLELKVVLLTVALVAYLVLFNVYPSWQWDCCGHSLGNLTGTN
801  GTLSSSSCSWHLKTMTNFYLVLFYTTLIMLSRQIDYYCRLDCLWKKKFKK
851  EHEEFETMENVNRLLLENVLPAHVAAHFIGDKLNEDWYHQSYDCVCVMFA
901  SVPDFKVFYTECDVNKEGLECLRLLNEIIADFDELLLKPKFSGVEKIKTI
951  GSTYMAAAGLSVPSGPENQDLERQHAHIGIMVEFSTALMSKLDGINRHSF
1001 NSFRLRVGINHGPVIAGVIGARKPQYDIWGNTVNVASRMESTGELGKIQV
1051 TEETCTILQGLGYSCECRGLIDVKGKGELRTYFVCTDTAKFQGLGLN
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Fig. 2. Amino acid sequence of bovine AC type 7 from bovine RPE as deduced from two identical full length cDNA clones. The 5′- and 3′- untranslated regions of the cDNA clone were 525 and 2,375 bp, respectively, excluding the poly(A)-tail of 26 A's (EMBL/GenBank accession # Z49806). Underlined amino acid sequences indicate predicted transmembrane regions. Met-1 and Met-20 which were used as transcription start sites, are marked in bold.

by subcloning and sequencing of the PCR product. This result indicated that these ACs from human and bovine RPE may be species variants of the type 7 AC isoform.

3.3. Cloning and sequencing of the bovine type 7 AC

Screening $8 \cdot 10^5$ plaques of the RPE-cDNA library with the above 996 bp AC fragment yielded 15 positive clones. Two full-length clones of 6.2 and 5.7 kb were completely double-stranded sequenced. Both had an identical ORF and coded for a novel type 7 AC of 1097 amino acids with a predicted molecular mass of 123.110 Da (Fig. 2). The clones only differed in the lengths of the 3′- and 5′-untranslated regions. A canonical Kozak sequence was absent. The cloned AC contained the usual doublet of six putative transmembrane spanning regions. Greatest similarity was to the type 7 ACs from mouse S49-lymphoma cells [10] and human KG1 leukemia cells [25,26] with 77% and 83% identity, respectively. Thus, the cloned RPE-AC belongs into a subfamily of ACs which comprises types 2, 4 and 7 isozymes which share a QXXER motif in the COOH-terminal region around amino acid residue 970. This sequence has recently been suggested to represent one of the potential sites for an interaction with the G-protein $\beta\gamma$ subunits [27,28]. Other structural features of the bovine type 7 AC isoform are the presence of several consensus sequences for phosphorylation by casein kinase 2, protein kinase C, tyrosine kinase and cAMP-dependent protein kinase.

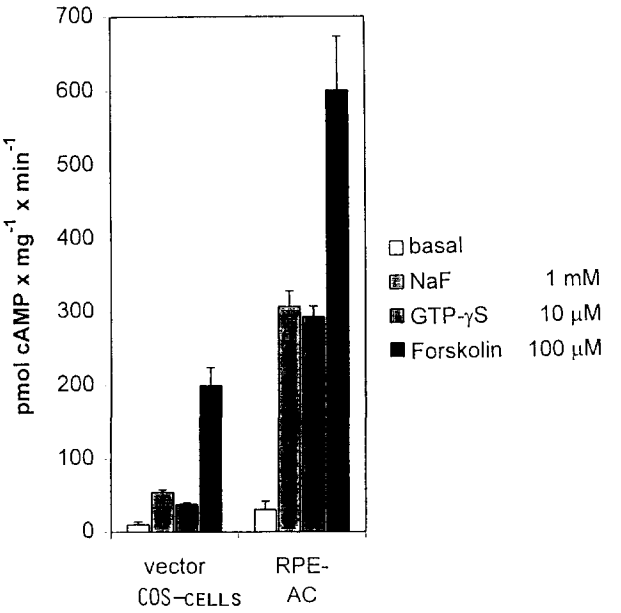

Fig. 3. Transient expression of the AC type 7 from bovine RPE in COS-1 cells. Met-20 was used as transcription start site. Membranes (30 μg) from control (pCMV5-vector alone) and pCMV5-AC-7 transfected COS-1 cells were assayed for AC activity as described in section 2.4. Values shown are the means of seven separate transfection experiments with duplicate determinations of enzyme activity.

3.4. Expression of bovine type 7 AC in COS-1 cells

To establish that the cloned cDNA encoded a functionally active AC it was attempted to express it in COS-1 cells directly from the cloning pBKCMV vector. This was unsuccessfull and may have been due to the absence of a Kozak sequence which also obscured the unequivocal identification of the translation start site. The bovine type 7 isoform carries a potential 19 amino acid NH_2-terminal extension compared to the other type 7 isoforms mentioned above (Fig. 2). Therefore, Kozak sequences were placed in front of both potential ATG start sites, the full-length clones were inserted into the pCMV5 expression vector and COS-1 cells were transfected [22]. Irrespective of the translation start sites, AC activity was expressed in both instances and basal activities from transfected cell homogenates were increased 3- to 4-fold compared to vector-transfected control cells (10 pmol cAMP/mg). Using the construct with Met at position 20 (bold in Fig. 2) as a transcripiton start, 100 μM forskolin, 1 mM NaF and 10 μM GTPγS stimulated basal activities 20-, 7.5- and 10-fold, respectively (Fig. 3). Comparable results were obtained with the 19 amino acids longer AC which used Met at position 1 for a transcription start (data not shown). We did not detect an effect of Ca^{2+} on the activity of the bovine type 7 isoform (tested range was 50 nM to 70 μM). These data agree with results obtained with other members of this AC subfamily, i.e. types 2 and 4 [2,4]. The presence of a forskolin-stimulated and G-protein-coupled AC was observed in homogenates from isolated bullfrog RPE [29]. Similarly, we detected in homogenates from isolated bovine RPE an AC activity which was stimulated by 100 μM forskolin, 1 mM NaF and 10 μM GTPγS 3-, 2- and 3-fold, respectively (data not shown).

4. Discussion

This is the first structural characterization of an AC specifically transcribed in the RPE. Its striking expression rate uniquely in this cell layer let us suggest that signal transduction via the type 7 AC is coupled to functions highly specific to the RPE. This layer of cells is of central importance in the functional maintenance and regeneration of the retina. It forms a highly differentiated monolayer of polarized cells situated at Bruch's membrane between the photoreceptors and the choriocapillaries with the apical villous processes interdigitating with the outer segments of rods and cones. The RPE controls the transport of ions, nutrients, and fluid to the retina. Further, the RPE is of utmost importance for the daily phagocytotic disposal of the outer segments of rods and cones. Due to the uniqueness of the RPE functions it is easily conceivable that it contains a predominant AC isoform which is specifically geared to these tasks. So far, studies on the role of cAMP in the RPE mainly used primary cell cultures. This introduces potential confounding factors as placing cells in culture can alter structural and functional characteristics. Nevertheless, many experiments with RPE cultures have convincingly implicated intracellular cAMP in the regulation of the electrical current crossing the RPE, in the transepithelial ion and fluid transport, and in the regulation of phagocytosis of the shed disks [29–31]. Some of these RPE functions appear to be regulated by light and, therefore, must be mediated by signals from the retina [34]. Receptors for adenosine [32,33], dopamine [34,35], adrenaline [36], vasointestinal peptide [37], and melatonin [38] have been reported to be coupled to the AC of the RPE and to affect the above mentioned processes.

The cloning of an AC which is by far the principal isoform in the RPE, will permit studies how these receptors are coupled to AC regulation, e.g. which G-proteins are involved. So far, enzymatic studies implicate $G_s\alpha$ and G_i in the regulation of the RPE-AC [38,39]. Yet, the presence of several more G-proteins in the RPE has been deduced by a cloning approach [40]. Finally, in view of the fact that a malfunction in the interaction of the retina and the RPE may be involved in some forms of hereditary retinal degeneration [41], it may be attractive to consider it as a potential target enzyme. Recently, the gene for the human AC type 7 has been localized to chromosomal region 16q12–16q13 [42]. While so far no gen-locus related to hereditary retinal diseases is known in this region [43] the Bardet Biedl syndrome, a disorder characterized by retinitis pigmentosa and other abnormalities, was linked to the adjacent region 16q21 [44]. The nearly exclusive expression of the AC type 7 in the RPE, the ease to prepare RPE or to use RPE cultured cells provide excellent conditions to study the specific physiological role of this isozyme.

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