Molecular cloning and expression of an adenylyl cyclase from *Xenopus laevis* oocytes

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Received 11 January 1997

Abstract **We have cloned a cDNA that encodes a novel *Xenopus laevis* oocyte adenylyl cyclase (xlAC) using oligonucleotides against conserved mammalian adenylyl cyclase regions. The isolated cDNA is 4372 bp long with an open reading frame of 4065 nucleotides which encodes a protein of 1355 amino acids. Comparison of the deduced amino acid sequence with previously cloned mammalian adenylyl cyclases shows a low identity, 19.7% with type 2 rat adenylyl cyclase and 24.2% with type 4 rat adenylyl cyclase, indicating that this *Xenopus* isoform represents a new member of this protein family. Gene expression studies of the xlAC by reverse PCR showed that this gene is expressed in all oogenesis stages but not during early embryogenesis. Expression of the xlAC in COS-7 cells resulted in increased basal AC activity, that was stimulated by forskolin, Gpp(NH)p and aluminium fluoride, and was insensitive to calcium and calcium-calmodulin (Ca^{2+}-CaM).**

Key words: Adenylyl cyclase; *Xenopus laevis* oocyte; cDNA cloning; Expression

1. Introduction

In eukaryotic cells, adenylyl cyclase (AC) is a widely distributed effector system regulated by G-protein coupled receptors (GPCR) which regulates the synthesis and levels of the second messenger cAMP. To date, eight different mammalian isoforms have been cloned and characterized from different sources [1–3]. All isoforms are stimulated by Gαs and forskolin but they differ in their tissue distribution and regulatory properties. Almost all AC, with the exception of type 4, are regulated directly or indirectly by Ca^{2+}. Types 1, 3 and 8 are activated by Ca^{2+}-calmodulin, type 5 and 6 are inhibited by Ca^{2+} levels and types 2 and 5 are activated by protein kinase C (PKC). Gβγ dimers also modulate the activity of some AC isoforms, activating AC types 2 and 4 and inhibiting type 1 [4,5].

All known mammalian AC isozymes share a predicted topological structure, designated as $NM_1C_1M_2C_2$, containing a short amino-terminal region (N) and two highly hydrophobic domains (M_1 and M_2) that are postulated to contain six transmembrane helixes and two cytoplasmic domains (C_1 and C_2).

In previous studies, we and other groups have biochemically characterized an AC activity present in the *Xenopus* oocyte plasma membrane and showed that this enzyme is activated by Gpp(NH)p, GTPγS, cholera toxin (CTX) and forskolin but is insensitive to Ca^{2+}-calmodulin [6–10]. Intracellular cAMP levels play an important role in the oocyte maturation process since inhibition of AC induces maturation [7] and substances that stimulate the enzyme block it [6,8]. In order to characterize this enzyme at the molecular level, we have cloned a full-length cDNA encoding a novel member of the AC family and expressed the protein in COS-7 cells.

2. Materials and methods

2.1. Isolation and sequencing of the xlAC cDNA

A 276 bp AC fragment was amplified by PCR using oligonucleotide primers based on conserved regions present in all mammalian AC [11]. The sense primer was 5′-AGATCAAGACAATCGGATCTACATACATGGC-3′ which corresponds to the amino acid sequence KIKTIGSTYMA and the antisense primer was 5′-CATACGAGAAGCCACGTTCACTGTGTTTCCCCA-3′ which encodes the amino acid sequence WGNTVNVAASRM. Phage DNA (100 ng), prepared from a λgt10 stage VI oocyte cDNA library [12], was subjected to amplification in 50 μl reactions containing 1×polymerase buffer; 1.5 mM $MgCl_2$; 200 μM of each dNTP; 400 nM of each primer and 2.5 units of Taq DNA polymerase (Promega Corp.). The reaction mixtures were incubated at 94°C for 5 min and then cycled 35 times for 1 min at 95°C, 1 min at 48°C and 1.5 min at 72°C, followed by a final incubation of 10 min at 72°C. The PCR amplified fragment was subcloned into the pGEM 3zf- vector (Promega Corp.) and sequenced by the chain termination method using [α-^{32}S]dATP and Sequenase version 2.0 (US Biochemical). The sequenced PCR product was then labelled by random priming, using [α-^{32}P]dATP and used to probe the xlAC cDNA library. Phages (5×10^5) were screened as described by Abramowitz et al. [13] and two positive phages were obtained, xlAC-a and xlAC-b. These clones were further purified through 3 rounds of consecutive screening. The cDNA inserts were excised with *Eco*RI, subcloned in the vector pTZ18R (Pharmacia) and sequenced as described above using successive oligonucleotides. Since the two isolated clones were truncated at their 5′ end, another 300 bp fragment, corresponding to the 5′ end of clone xlAC-b, was amplified and labelled to rescreen the library. 1×10^6 phages were analyzed, from which four positive clones were obtained. Again, the phages were further purified and the cDNAs subcloned and sequenced as described above.

2.2. Sequence comparison analysis

Data analysis software CLUSTAL [14] was used to compare mammalian and *Xenopus* cloned AC. Mammalian AC sequences were obtained from the Gene EMBL database.

2.3. Reverse PCR

Total RNA was prepared from isolated oocytes stages I to VI and from morula and blastula embryonic stages using guanidinium thiocyanate as described by Chomczynski and Sacchi [15]. Total RNA (1 μg) was reverse transcribed with the antisense primer 5′-TCAGACATTATGAAAAGATG-3′ (cDNA position 1318) according to Oñate et al. [16]. A 20 μl volume of the reverse transcription reaction

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The nucleotide sequence reported here has been submitted to the GenBank/EMBL Data Bank with the accession number Z46958.

PII S0014-5793(97)00100-2

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MEIQVTGVSGQARSCQNPSVCLKFTAGGVSRGGGGELVGGKVPDKLYINNMASPVNQQLL    60
HHTEVRCDGSGDGSSVTVRINRQHHQAPSRRCKYSISSSCSSGESGVKKTGGSGGARRQK   120
KLPQLFERSTSNWWNPKFDSNNLEEACVERCFPQTQRRFRYALMYLSVAGLLWSIYFSVH   180
MKTKLVSHLVPTLCFLIVCLGFFFFTFTKSYARRHCTAISLLVTLLVFTLTLASQFQVLN   240
GLGSDSLSNLTSFSATGSSSCLSQVGSFSICVEVLLLLYTVMHLPLYLSACLGVAYSILF   300
ETFGYHFRDESCFVLLVGRMAHWELLSKALLHVCIHAIGVHLFIMSEVRSRSTFLKVGQS   360
IMHGKDLEVEKALKERMIHSVMPRIIADDLMKQGDDESENSVKRHSASSPKSRKKKSSIQ   420
KTPIIFRPFKMQRIEQVSILFADIVGFTKMSANKSAHALVGLLNDLFGRFDRLCEETKCE   480
KISTLGDCYYCVAGCPEPRPDHAYCCIEMGLGMIEAIDQFCQEKKEMVNMRVGVHTGTVL   540
CGILGMRRFKFDVWSNDVNLANLMEQLGVAGKVHISEKTARYLDDRYLMEDSMVVERLGQ   600
IVAADQLKGLKTFLISGGRTRVPSCSCSQTLIPVQEGTDLSSPSLAPHVQAAISETSDSH   660
TNCTQPETLKSCPSCGETAARDGPEEGVSAANGGGEEWKGGAPRPSAIGASLKDPERSPE   720
SSTGDTLTNSQASLYDMLQEKGRWCGVSMDQSALLPLRFKNIREKTDAHFVEVIKEDSLM   780
KDYFFKPPINPLSLNFLDKELETSYRASYQEEVIRMAPVKTFASATFSSLQDVLLNYFIF   840
VLLSVACLLKPGTNTVSPPTLALVLLSVCGLLGFLSLLVSVRMAFYLEDMLLCTRRLLEI   900
ISGWVPRHFIGTVLVCLPAAVIFSYLSSDFYTDIHYTMFLCSALLIPMVQYCNFCQLSSS   960
ALLLATITGATMLILIYLPLCPQRPPLDPGTDIEANLSTSNSSYETLDNPRTELPFTRLG  1020
QEIAVAYFLLLLLVWFLNREFDVSYRLHYHGDVEADLHRTKIQSMRDQADWLLRNIIPYH  1080
VAEQLKVSQSYSKNHDDAGVIFASIVNFSEFYEENYEGGKECYRALNELIGDFDELLSKP  1140
HYSCIEKIKTIGATYMAASGLNPSQCQDSSQPHRHLQTLFEFAKEMMSVVDEFNNNMLWF  1200
NFKLRIGFNHGPLTAGVIGTTKLLYDIWGDTVNIASRMDTTGVECRIQASEESYRVLVKM  1260
GYDFDYRGTVNVKGKGQMKTYHFPKCTDNGGLVPHHQLCISPDIRVQVDGSIGRSPTDEI  1320
SSLVTGGKGAVELGSGEAERKREKAEERGRDGGAR                            1355
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Fig. 1. Deduced amino acid sequence of the *Xenopus laevis* oocyte cDNA clone. Sequences, predicted as transmembrane regions, are underlined and empty boxes indicate the two amino acid conserved regions present in all mammalian adenylyl cyclases. Numbers on the right indicate the amino acid position along the sequence.

was mixed with 80 μl of a PCR master mixture containing the sense primer 5-TGTGGAGCATCTACTTTAGT-3′ (cDNA position 812) and the rest of the PCR mixture components [16]. Aliquots of 10 μl of the PCR reaction were subjected to electrophoresis on 2% Nusieve-1% agarose gels in the presence of ethidium bromide.

2.4. Adenylyl cyclase expression in COS-7 cells

The reconstructed full-length xlAC cDNA was subcloned into the pCEV29 vector to give the recombinant vector pCEV29/xlAC. COS-7 cells were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with inactivated 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified incubator containing 5% CO_2 at 37°C. Transfections were performed using 5 μg of plasmidial supercoiled DNA by the DEAE-dextran method, including chloroquine treatment. After 2 days, cells were scraped in lysis buffer (25 mM Tris-HCl, pH 7.5; 1 mM EDTA, 1 mM DTT; 0.1 mg/ml leupeptin and 1 mM PMSF) and homogenized by passing the cells 10 times through a 23-gauge needle. Cell lysates were immediately assayed for AC activity.

2.5. Adenylyl cyclase assay

AC activity was measured at 32°C in a buffer containing 25 mM Tris-HCl pH 7.5, 1 mM 3-methylisobutylxanthine (IBMX), 5 mM $MgCl_2$, 1 mM EDTA, 20 mM creatine phosphate, 250 μg/ml creatine phosphokinase, 30 μg/ml myokinase, 1 mM cold cAMP, 1×10^4 cpm [^{3}H]cAMP, 0.1 mM ATP and 5×10^6 cpm [α-^{32}P]ATP. The reaction was started by the addition of 20 μg of cell lysate to a final volume of 50 μl. All points were assayed in triplicate and cAMP accumulation was determined by Dowex and alumina chromatography as described by Birnbaumer et al. [17]. Protein concentration was measured by the method of Lowry et al. [18].

3. Results

3.1. Cloning of a cDNA encoding a complete open reading frame (ORF) of a Xenopus laevis oocyte adenylyl cyclase

A 4372 bp cDNA fragment encoding the complete amino acid sequence for an *X. laevis* oocyte adenylyl cyclase was isolated from a stage VI *Xenopus* oocyte cDNA library. The first screening gave two positive clones, xlAC-a and xlAC-b, that were truncated at the 5′ end region (NH_2-terminal). In order to clone the complete cDNA, a second screening was performed using a PCR probe corresponding to the 5′ end region of clone xlAC-b. Four positives clones were obtained and the largest one, clone xlAC-d (3200 bp) was truncated at its 3′ end but overlapped with clone xlAC-b by 1042 bp. The overlapping region contained a *Bam*HI site which was used to generate the complete cDNA from clones xlAC-b and xlAC-d. The resulting sequence was 4372 bp long with a putative initiation codon at position 300 and a stop codon at position

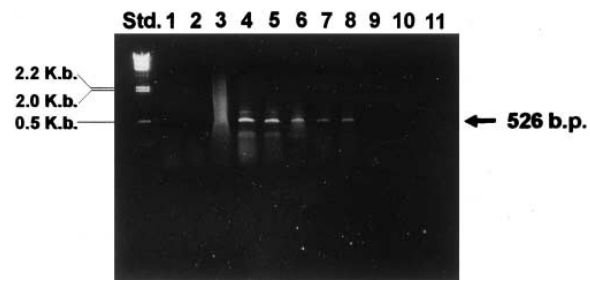

Fig. 2. Reverse PCR detection of xlAC mRNA from different oocyte stages and early embryogenesis. 1 μg of total RNA from oocyte stages I to VI and from morula and blastula were subjected to reverse PCR amplification with the specific pair of primers indicated in Section 2. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The arrow indicates the size of the xlAC product (526 base pair). Lanes: 1, negative control without RNA; 2, negative control without RNA and the sense primer; 3, negative control without the sense primer; 4, oocyte stages I and II RNA; 5, oocyte stage III RNA; 6, oocyte stage IV RNA; 7, oocyte stage V RNA; 8, oocyte stage VI RNA; 9, blastomer RNA; 10, morula RNA; 11, blastula RNA.

4364. No polyadenylation signal and no poly-A tail were found at its 3′ end.

3.2. Analysis of the xlAC predicted amino acid sequence

The cloned cDNA has an open reading frame that encodes a protein of 1355 amino acids with a calculated molecular mass of 150 kDa (Fig. 1). Hydropathy analysis of the xlAC predicted amino acid sequence showed the presence of 12 transmembrane spanning regions (data not shown), a characteristic feature for all adenylyl cyclases (Fig. 1, underlined sequences). Alignment of the xlAC protein sequence with other known mammalian species showed in general, a low identity (24% with bovine type 1; 23% with rat type 2; 20% with rat type 3; 22% with rat type 4; 19% with mouse type 5; 21% with rat type 6 and 22% with rat type 8 adenylyl cyclases). Recently, a mouse type 9 adenylyl cyclase has been reported [19,20] which is more similar to the xlAC (70% of identity), and the dendrogram obtained from this alignment clearly shows that these two isoforms represent a new group of this protein family (data not shown).

3.3. Detection of xlAC mRNA during oogenesis and early embryogenesis by the reverse PCR reaction

Expression of xlAC mRNA was examined by reverse PCR using specific xlAC antisense primers and total RNA from oocyte stages I to VI and from morula and blastula embryonic stages. A 526 bp gene fragment was amplified in all oocyte stages but not in early embryonic stages (Fig. 2). The decreasing amount of xlAC mRNA observed through stages I to VI is due to the increase in the total RNA mass per oocyte.

3.4. xlAC expression in COS-7 cells

In order to determine whether the xlAC cDNA indeed encoded a functionally and active enzyme, COS-7 cells were transfected with the pCEV29/xlAC vector and AC activity was measured. Fig. 3 demonstrates the kinetics of basal and forskolin AC activation of transfected COS-7 cell lysates. The

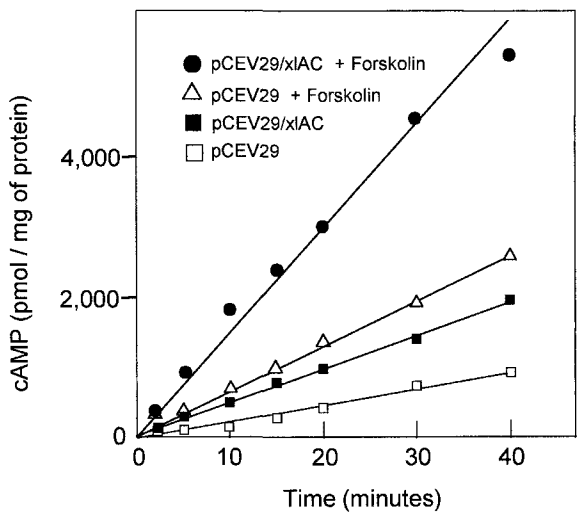

Fig. 3. Kinetics of cAMP accumulation. Lysate fractions from COS-7 cells transfected with the plasmid pCEV29 alone (□, △) and with the construction pCEV29/xlAC (■,●) were incubated in the absence (□,■) or presence (△,●) of 100 μM forskolin. 50 μl aliquots (20 μg of protein) were taken at the indicated times and cAMP production was determined as described in Section 2.

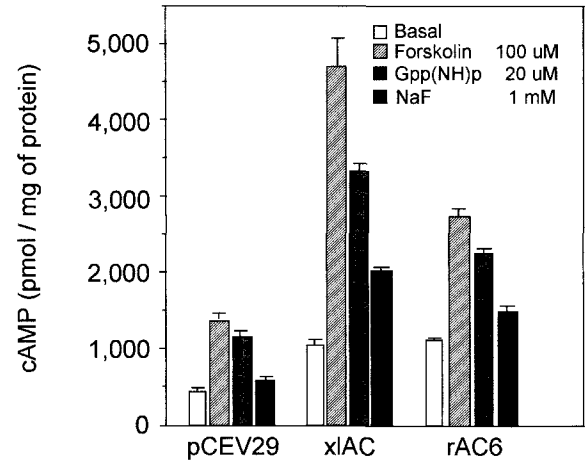

Fig. 4. Induction of cAMP levels by known AC effector molecules. 20 μg of crude lysate from COS-7 cells transfected with the plasmid vector pCEV29 alone, with xlAC or with rat AC6, were incubated with 100 μM forskolin, 20 μM Gpp(NH)p and 1 mM NaF for 30 min and cAMP production was determined as described in Section 2. The data shown are the mean ± S.D. of three experiments.

basal activity was about 3-times higher in cells transfected with the pCEV29/xlAC vector when compared to those transfected with the vector alone, and this difference was even more dramatic when 100 μM forskolin was used. To study the regulatory properties of the xlAC, enzymatic activity was determined in the presence of different effectors. As shown in Fig. 4, basal activity of xlAC transfected COS-7 cells was stimulated 4.7-, 3.3- and 2-fold by 100 μM forskolin, 20 μM Gpp(NH)p and 1 mM NaF, respectively. As a control, we transfected cells with rat AC6, which showed a similar response, but the level of stimulation with the different activators was lower. After proving that the xlAC was active and responded to the different effectors, we studied the role of Ca^{2+} in the regulation of xlAC activity. As shown in Fig. 5, no effect on xlAC and mouse AC9 was seen on addition of 1 mM EGTA, 1 μM Ca^{2+} or 1 μM calmodulin plus 1 μM Ca^{2+}. In contrast, rat AC8 was inhibited by EGTA and stimulated by Ca^{2+} and Ca^{2+}/calmodulin as has been described by Seamon and Daly [21].

4. Discussion

In the present study we cloned the xlAC using two primers against two highly conserved regions present in the second cytoplasmic loop of all mammalian AC [11]. Subsequently, these primers were used to amplify a DNA fragment to screen the *Xenopus laevis* cDNA library. After two consecutive screenings it was possible to isolate two truncated clones that overlapped by a region of more than 1000 bp which contained a single *Bam*HI restriction site that was used to construct the full-length xlAC gene. By analyzing the deduced amino acid sequence, we concluded that the ATG localized at position 300 within the sequence GCATGGAG, that is similar to the Kozak consensus sequence, is the putative translation initiation codon. Although this 5′ region has a relatively low GC content (< 50%), it contains seven stops codons that are upstream of the ATG mentioned above. The open reading frame, which starts at this point, is 4065 bases long and encodes a protein containing 1355 amino

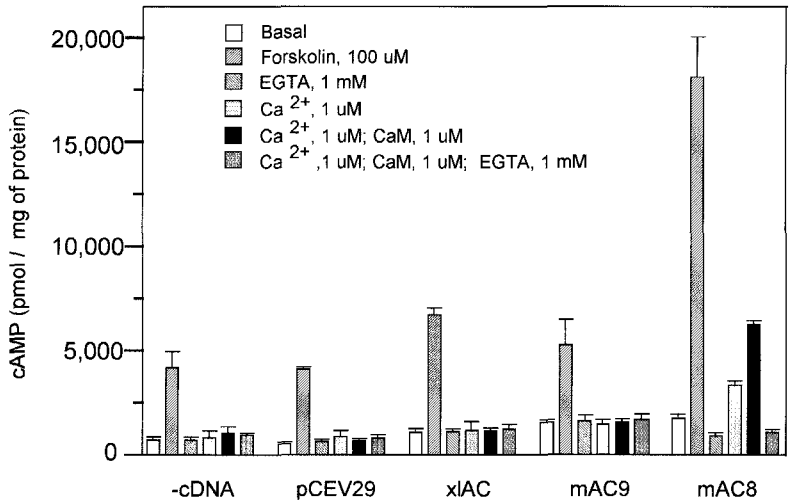

Fig. 5. Effect of Ca^{2+} and calmodulin on xlAC, mouse AC9 (mAC9) and mouse AC8 (mAC8). 20 μg of the crude lysate fraction from non-transfected COS-7 cells and from cells transfected with the vector pCEV29 alone, xlAC, mAC9 and mAC8 were incubated with Ca^{2+} and Ca^{2+} plus calmodulin in the presence or absence of EGTA for 30 min and cAMP production was determined as described in Section 2. The data shown are the mean ± S.D. of three experiments.

acids. Hydropathy analysis showed that this protein shares the same topological distribution of all mammalian ACs and also contains the two highly conserved sequences EKIKTIGATYMA and YDIWGDTVNIASRM. The large divergence observed between xlAC and other mammalian ACs indicates that, together with rat type 9 AC, they belong to a new group within this protein family.

Detection of xlAC mRNA during oogenesis agrees very well with previous studies that biochemically characterized this activity in the *Xenopus* oocytes [22]. The oocyte AC activity shares the same regulatory properties with the protein encoded by the xlAC gene characterized in this work, since it is activated by forskolin, Gpp(NH)p, aluminium fluoride and insensitive to Ca^{2+} and Ca^{2+}/calmodulin.

In *X. laevis* oocytes, progesterone induces maturation through a still unidentified membrane receptor, and its first effect is to inhibit AC which causes a decrease in intracellular cAMP levels [20]. For this reason it is important to continue characterizing this xlAC to understand its physiological role in the maturation process.

Acknowledgements: This study was supported by Grant No. 1940256 from FONDECYT. We also would like to thank Dr. Richard Premont (Duke University Medical Center, Durham, NC, USA) for type 6, 8 and 9 AC cDNAs and Lauren Jean Aguayo for critical reading of the manuscript.

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