In Vivo Generation of an Adenylyl cyclase Isoform with a Half-molecule Motif*

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A truncated form of adenylyl cyclase (type V-α) has been cloned from a cardiac cDNA library. It constitutes a half-molecule of type V adenylyl cyclase diverging at the end of the first cytoplasmic loop. Northern blotting study has revealed the presence of such a mRNA species (approximately 3.5 kilobases in size) in the heart. Genomic sequence analysis has revealed that type V-α is generated via usage of a polyadenylation signal located within an intron sequence of type V adenylyl cyclase gene. When type V-α is co-expressed with an artificially generated half-molecule constituting the latter half of type V adenylyl cyclase, the catalytic activity in transfected cell membranes is significantly higher than that of controls. However, when either alone is overexpressed, no significant increase in catalytic activity results. These results indicate that a half-molecule of adenylyl cyclase, i.e. a protein containing six-transmembrane spans followed by a single cytoplasmic domain, can be generated in vitro, but catalytic activity is lacking unless heterodimerization can occur. This finding identifies another potential mechanism for generating diversity within this enzyme family.

Molecular cloning studies have identified a multigene family of mammalian adenylyl cyclases. So far six different isoforms have been isolated (types I to VI) (1-8). Although different in amino acid sequence, biochemical characteristics, and tissue distribution, they share a common structure of six-transmembrane spans followed by a large cytoplasmic domain repeated in tandem. Although the amino acid sequence differs significantly in the transmembrane regions among the isoforms, that of the cytoplasmic and putative catalytic domains is relatively well conserved (9). Indeed the first and the second cytoplasmic regions are similar to each other (6). These regions also show homology to the C-terminal, catalytic domain of mammalian guanylyl cyclases (1). We have recently described a dendrogram based upon the homology score among the various mammalian adenylyl cyclase family members (6). Following divergence from guanylyl cyclase, the adenylyl cyclase types I and III diverged from the others; thereafter types II/IV and V/VI diverged forming subgroups within the family.

The guanylyl cyclase family contains members that exist in both the particulate and soluble fractions of cell homogenates. Soluble forms of the enzyme recognize nitric oxide or its related products, while diversity within the extracellular domain of the plasma membrane forms has resulted in a series of guanylyl cyclases that are activated by different peptide ligands, including atrial natriuretic factor (10, 11). However, both forms possess a C-terminal, catalytic domain, whose sequence is homologous among all the family members. Soluble forms of guanylyl cyclase have two distinct subunits, α and β (12-14). Although each subunit contains an apparent catalytic domain, it does not function independently; previous studies have suggested that heterodimer formation is necessary (13). This finding is consistent with recent observations on the calmodulin-sensitive form of adenylyl cyclase (type I) found in brain. It has been shown that artificially created molecules, constituting either the initial or latter half of the original molecule, must be expressed together to generate catalytic activity (15).

We have now cloned a novel type V adenylyl cyclase cDNA; it contains the first half of the molecule while totally lacking the latter half and instead terminates in a short stretch of novel amino acid sequence. In addition to its cloning, we also describe the putative mechanism for generating such a molecule.

MATERIALS AND METHODS

Isolation and Sequencing of Clones—A cDNA library was prepared according to standard procedures in a λgt10 vector using poly(A)* RNA prepared from canine ventricular tissue (16). In the primary library screening, 2.0 × 10⁶ independent clones were screened with an EcoRI-SphI fragment from type V adenylyl cyclase cDNA as a probe. The hybridization was carried out in a solution containing 50% formamide, 5 × SSC, 5 × Denhardt’s solution, 25 mM NaPO₄ (pH 6.5), 0.25 mg/ml calf thymus DNA, and 0.1% SDS at 42 °C. Hybridization was carried out in the same buffer at 42 °C for 14-20 h with cDNA fragments labeled with ³²P, followed by washing under increasingly stringent conditions. All DNA sequencing (using either universal or synthetic oligonucleotide primers) was carried out bidirectionally at least twice using either Sequenase (17) or Taq polymerase (18). For certain GC-rich sequences, such as the 5'-untranslated region, the reaction was carried out both with Sequenase and Taq polymerase with or without 7-deaza-dGTP (19). Electrophoresis was carried out in a polyacrylamide gel containing 8% urea and 20% formamide, when necessary, to eradicate problems with band compression. For genomic DNA cloning, 2 × 10⁶ recombinant clones from an EMBL3 canine genomic DNA library were screened with a 0.4-kb AatII fragment as a probe that contained the unique sequence from the type V-α cDNA. Screening and sequencing were subsequently performed in a manner analogous to that described above.

Transient Expression in CMT Cells—A 2.1-kb, EcoRI-Stul fragment, designated as 113-α (for type V-α), was constructed by ligating the EcoRI-Stul fragment (nucleotide 1-734) from clone 72 in pUC18, SphI-EcoRI (nucleotide 954-1604, from clone 7), and EcoRI-Stul (nucleotide 1604-2137, from clone 6L) fragments. A 2.8-kb, EcoRI-SphI fragment was inserted into the KpnI site of pRC1, a canine fibrosarcoma cell line, to give pRC1/S. This plasmid was transfected into CMT cells, and total RNA was extracted and analyzed by Northern blotting.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) M97886.

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†‡The abbreviations used are: kb, kilobase(s); bp, base pair(s); GTPγS, guanosine 5′-O-(thio)triphosphate.
fragment designated as 113-β (for type V-β) was constructed by digesting clone 113 with EcoRI and SspI (nucleotide 1639–4033 from type V adenyllylcyclase) (5). There is a Met at amino acid residue 581 with a reasonable Kozak consensus sequence (...)CCAGATGA(...) in 113-β; thus it would translate a protein of 604 amino acids constituting the latter half of type V adenyllylcyclase starting in the Clb domain. Each fragment was subcloned into unique polylinker sites of the plasmid pcDNA I-amp and designated pcDNA113α and -β, respectively. Twenty micrograms of the purified plasmid were transfected into CMT cells (20) by the method previously described (21). Control cells were mock-transfected and were otherwise treated in the same way.

Northern Blotting—A 60-mer antisense oligonucleotide probe was synthesized based on the novel sequence of 6L (AGCCGTCCCGGA ATCAG~GGGCCCTTCCTTACAAAGAACCGTAGGCGAAGGA) synthesized based on the novel sequence of 6L (AGCCGTCCCGGA ATCAG~GGGCCCTTCCTTACAAAGAACCGTAGGCGAAGGA AGAACAG) (Fig. 1b) and was labeled with 32P. Poly(A) mRNA was isolated from canine heart tissue by standard techniques using oligo(dT)-cellulose. Hybridization was performed as described above, except that a 30% formamide solution was used.

Adenylylcyclase Assay—The transfected CMT cells were washed twice with phosphate-buffered saline and then collected into 1 ml of cold buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 10 μM phenylmethylsulfonyl fluoride, 100 units of leupeptin, and 50 units of egg white trypsin inhibitor. The cells were homogenized with a Polytron (setting 6 for 10 s) and centrifuged at 800 × g for 10 min at 4 °C. The supernatant was further centrifuged at 100,000 × g for 40 min at 4 °C. The resultant pellet was resuspended in 50 mM Tris (pH 8.0), 1 mM EDTA, 1 μM phenylmethylsulfonyl fluoride, 50 units of leupeptin, and 50 units of egg white trypsin inhibitor to a concentration of 5 μg/μl. This crude membrane preparation was used in the adenylylcyclase assay.

Adenylylcyclase activity was measured by the method of Salomon (22). Briefly, the washed membranes from CMT cells (5–10 μg/assay tube) were resuspended in 100 μl of solution containing 4 mM Hepes (pH 8.0), 2 mM MgCl2, 0.1 mM cyclic AMP, 0.1 mM ATP and 32P ATP (0.2–5 μCi/assay tube), 1 mM creatine phosphate, 8 units/ml creatine phosphokinase. The reaction mixture was incubated at 30 °C for 30 min and the reaction was stopped by the addition of 100 μl of 2% SDS. To monitor the recovery from the columns, 3H-labeled cyclic AMP was used. Cyclic AMP was separated from ATP by passing through Dowex and alumina columns, and the radioactivity was measured by liquid scintillation counting. Protein concentration was measured by the method of Bradford (23) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Isolation of Type V-α Clones—We obtained 12 primary cDNA clones using an EcoRI-SphI probe from type V adenyllylcyclase. Each was subcloned into pUC18 and sequenced. Eleven of the 12 cDNAs were identical to type V adenylylcyclase (5). There is a Met at amino acid residue 581 but totally diverged at its 3'-end; it contained 570 amino acids, 25 of which (residues 572–596) are unique to this isoform. These data suggest that during the processing of the precursor RNA a cryptic polyadenylation signal utilized in this isoform is not the most preferred one, likely contributing to the relatively small amount of mRNA which is generated (27).

mRNA Expression—In order to confirm that the mRNA encoding this unique isoform is indeed expressed, Northern blotting was performed using a 60-mer antisense oligonucleotide designated as the latter half of type V-α adenylylcyclase (Fig. 1b). The unique sequence found in clone 6L was located adjacent to an exon-intron boundary identified within the genomic fragment and in fact spanned the consensus sequence of the 5'-donor splice site (AAGC CCGG) (25).

These data suggest that during the processing of the precursor RNA a cryptic polyadenylation signal within the intron, 260 bp downstream from the conventional exon-intron border, can be utilized to generate a novel truncated mRNA (26). Thus the mRNA of this novel form of adenylylcyclase, designated type V-α, incorporates a part of the intron into the 3'-terminal sequence (Fig. 2). The consensus sequence for polyadenylation utilized in this isoform is not the most preferred one, likely contributing to the relatively small amount of mRNA which is generated (27).
otide whose sequence is unique to clone 6L as a probe (Fig. 1a). The results indicate that such a mRNA species (approximately 3.5 kb in size) does exist in heart (Fig. 3). For comparison, the conventional isoform has two apparent splicing variants, 5 and 7 kb, as previously reported (5).

**Expression in CMT Cells**—The adenylyl cyclase activities of three different constructs were compared: pcDNA113-72, which encodes the conventional, full-length molecule of type V adenylyl cyclase; pcDNA113-α, which encodes the initial half of the protein (type V-α); and 113-β, which encodes the latter half of the protein (type V-β). When expressed alone, neither pcDNA113-α nor pcDNA113-β had increased activity over the mock-transfected control. However, when co-transfected together, the activity was significantly enhanced. In the presence of forskolin, there was a 1.6-fold increase over the mock-transfected control, although this activity was less than half of that observed with the conventional type V adenylyl cyclase (Fig. 4).

The above data suggest that type V-α adenylyl cyclase alone cannot catalyze the conversion of ATP to cyclic AMP. However, when co-expressed with the latter half of the molecule, this function is restored, implying that, at the very least, heterodimerization of the two molecules is required. A similar mechanism of catalytic activation requiring heterodimerization of distinct subunits has previously been described for the soluble form of guanylyl cyclase which shares sequence homology to adenylyl cyclase in its catalytic domain.

There is a superfamily of transporter molecules including P-glycoprotein (28), the cystic fibrosis gene product (29), and major histocompatibility complex-encoded peptide transporters (30), which utilize ATP for energy and share the common motif of tandem repetition of six-transmembrane spans followed by a large cytoplasmic domain. Although adenylyl cyclase neither shows homology in its putative catalytic domains to this family of molecules nor does it possess an ATP-binding consensus domain, it does share the same molecular topology as these other transmembrane proteins. Several studies have suggested that the interaction of distinct catalytic domains either contained in the same molecule or in discrete subunits may underlie the catalytic activation of this class of molecules. An example of the latter is provided by RING4 and RING11, two major histocompatibility complex-encoded peptide transporters, which are the products of distinct genes. Apparently these two proteins assemble to form a complex since a defect in either protein results in the formation of an unstable molecule and loss of function (31).

Our data suggest that a single gene encoding an adenylyl cyclase isoform can generate either a full or half-molecule motif via usage of alternative polyadenylation signals. The findings that such a half-molecule does exist for type V adenylyl cyclase has certain implications for the generation of functional diversity in the signal transduction pathway culminating in adenylyl cyclase activation. In addition to the existence of multiple isoforms (some of which appear to be expressed in the same cell type) of adenylyl cyclase, which facilitates diversity in signal transduction, it is possible that half-molecules of different adenylyl cyclase isoforms might also be encoded, functionally couple, and thereby create heterodimers with novel properties. Gilman and co-workers (15) have published data indicating that co-expression of artificial half-molecules of type I and type II adenylyl cyclase can produce, putatively via heterodimerization, a novel adenylyl cyclase activity that possesses the βγ sensitivity of type I yet maintains the other characteristics of type II adenylyl cyclase. We have, as yet, no evidence that other adenylyl cyclase genes (Type I-IV and VI) either via the mechanism described in this study or by other routes (e.g. alternative promoters) can generate such half-motif adenylyl cyclase molecules.

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