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Two domains are critical for the nuclear localization of soluble adenylyl cyclase

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Abstract

Soluble adenylyl cyclase (sAC) is a newly identified source of cyclic adenosine 3′,5′-monophosphate (cAMP). Unlike the well-known transmembrane adenylyl cyclases (tmACs), sAC locates to the nucleus, mitochondria and microtubules. For most cAMP-signaling microdomains, there is always an AC nearby, for example tmAC. But it was until the discovery of sAC that there was not known cAMP resource in the nucleus. sAC associates with nuclear cAMP-signaling microdomains, which were once considered to depend on the diffusion of cAMP produced by tmAC. In this report, we focus on the truncated soluble adenylyl cyclase (tsAC), the most common existence form of sAC in tissues. Two domains (145–200 aa and 257–318 aa) related with sAC nuclear localization were present here. The findings provide evidence that these two domains are critical for the nuclear localization of sAC and they collocated with the catalytic domains.

Keywords: sAC; tsAC; cAMP-signaling microdomain; Nuclear localization; Immunofluorescence

1. Introduction

Cyclic adenosine 3′,5′-monophosphate (cAMP), the product of adenylyl cyclase (AC), is an important second messenger that affects a multitude of cellular functions in almost all prokaryotic and eukaryotic organisms [1,2]. A previous model of cAMP signaling requires the diffusion of cAMP from cell membrane to the targets throughout the cell. Obviously, diffusion of cAMP would likely diminish specificity and selectivity [3]. Moreover, the existence of phosphodiesterases (PDE), which is responsible for cyclic nucleotide hydrolysis, limits the spread of cAMP [4].

The theory on cAMP action in subcellular microdomains was first put forward about 20 years ago [5]. Recently, along with the development of new techniques for assaying cAMP [6], there is more and more evidence to support this theory. First, the same extracellular signal may result in different cAMP signals in different cellular compartments [6–9]. Second, those proteins involved in the same cAMP-related signal transduction cascade tend to colocalize in the same microdomain [10,11]. For example, in beta(2)-adrenergic receptor cascade, those related proteins, such as G-protein, AC, the C L-type calcium channel, form a signaling complex [11–14].

Although the compartmentalization of cAMP signaling near cell membrane has been supported by much direct evidence, the research of the cAMP-related cascade inside the nucleus still follows behind [15]. For a very long time, transmembrane adenylyl cyclases (tmACs), which locate on cell membrane, are the only known source of cAMP [16]. The phosphorylation of cAMP response element binding protein (CREB) in the nucleus is considered to depend on the diffusion of cAMP or C subunit of protein kinase A (PKA) [17]. The recognition of soluble adenylyl cyclase (sAC) gives a new explanation. SAC has been proved to take part in CREB activation and forms a nuclear cAMP microdomain [18].

sAC is a newly described protein with the activity of AC [19]. Unlike tmACs, sAC is G-protein insensitive and is uniquely responsive to bicarbonate, the concentration of which will reflect the change in pH or CO_2 [20]. Without identifiable transmembrane domain, the localization of sAC is far away from tmAC. SAC is distributed throughout the cell and associates with distinct cellular compartments [21]. Immun-

Abbreviations: AC, adenylyl cyclase; cAMP, cyclic adenosine 3′,5′-monophosphate; NE, nuclear envelope; NLSs, nuclear localization signals; sAC, soluble adenylyl cyclase; tsAC, truncated soluble adenylyl cyclase.

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Table 1
Primers used to obtain the cDNA of tsAC

	Forward primer	Reverse primer
Fragment a	5′-TCCTTGAACATGAGTGCCCGAA	5′-CCGAAGCTGCTTGTCATCAATCTG
Fragment b	5′-AACTTCCTAAGGCTTGCCTGCATG	5′-CTAACACGACACTTTCTCATTGAGGCC

ofluorescent staining demonstrates that sAC is located to the nucleus, mitochondria and microtubules [21]. SAC has also been proved to be the only functional form of AC present in the nucleus [18,22]. The discovery of sAC inside those microdomains without known cAMP source perfects the cAMP-signaling compartmentalization theory. As mentioned above, sAC produces cAMP near the effecter in the nucleus and forms a nuclear cAMP-signaling microdomain.

In mammals, two kinds of transcripts encode full-length and truncated soluble adenylyl cyclase (tsAC) [23]. For lacking of Exon 11, the truncated transcript has a premature termination of open-reading frame after the catalytic domains [23]. Both the full-length and the tsACs are detected in germ cells, where they contribute to the AC activity. Though sAC is only highly enriched to male germ cells [24], tsAC exist in almost all tissue being tested [22,25]. Furthermore, the truncated form was the only one that could be detected by Western blot in nine eukaryotic cell lines (data unpublished).

For most proteins located inside the nucleus, a nuclear localization signals (NLSs) is always needed to lead the protein entering the nucleus [26]. Usually, NLSs comprises a short stretch of basic amino acid. With the help of importin, NLSs could lead the protein through nuclear pore [26]. For most components of cAMP signaling, they either include localization sequence (like tmAC) or have other proteins tether them to special domains (like A-kinase anchoring proteins and PKA). But searching of NLSdb [27], a database of NLSs (http://cubic.bioc.colubia.edu/db/NLSdb) indicated no putative sequence in tsAC taking charge of its nuclear localization.

Here, we focus on the 48 kDa tsAC. Multiple mutants were constructed and expressed in eukaryotic cells. Their cellular locations were observed one by one. Based on these results, we presented here that two domains of tsAC related to its nuclear localization. Deletion of these sections results in changes in tsAC localization.

2. Materials and methods

2.1. Materials

Human cervical carcinoma HeLa cell-line (maintained in our lab); Ex Tag (TakaRa Biotechnology Co., Ltd); Restriction Enzyme *Xho*I, *Eco*RI, *Sac*I and *Xba*I (TakaRa Biotechnology Co., Ltd); T4 DNA ligase (TakaRa Biotechnology Co., Ltd); TRIZOL Reagent (Life Technologies, Inc); SuperScript first-strand synthesis system (Life Technologies, Inc); vector pGBKT7 (Clontech Laboratories, Inc.) pEGFP-C1 (Clontech Laboratories, Inc.) and pCDNA6/V5-His-HA (a kind gift from Professor Wanglinfang). The HA-tag version of pcDNA6/V5-His (Invitrogen) was generated by inserting the HA-tag in *Nhe*/*Hin*dIII sites of pcDNA6/V5-His version B (invitrogen).

2.2. RNA extraction and RT-PCR

Total RNA was extracted from testis of male Harlan Sprague-Dawley rat using Trizol Reagent (Life Technologies, Inc.) following the manufacturer's protocol and then precipitated with cold ethanol. The dried pellet was then dissolved in ribonuclease-free H_2O for RT-PCR.

To obtain the cDNA of tsAC, up to 5 μl total RNA was reverse-transcribed by using SuperScript first-strand synthesis system (Life Technologies, Inc). According to the sAC sequence supplied by NCBI, two groups of primers (see Table 1) were designed to get different parts of tsAC, fragment a and fragment b, which could be ligated by an internal *Xho*I site (Fig. 1) to get the tsAC. PCR was performed directly on 2 μl of first-strand cDNA with 800 nM each of primers (see Table 1).

2.3. Plasmid construct

The cDNA of tsAC was ligated with *Eco*RI, *Pst*I linearized pGBKT7 vector. In this study, mutagenesis was performed with a PCR-based strategy on tsAC-pGBKT7 as a template. The tsAC and its multiple mutants were constructed into two kinds of eukaryotic expression vectors, pEGFP-C1 and pcDNA6/V5-His-HA. For MutG0, a1-a5,b1-b6, different length of tsAC cDNA was amplified with primers (see Table 2) containing *Sac*I and *Eco*RI restriction site (*italic*). Purified PCR products were digested and ligated into *Sac*I and *Eco*RI linearized pEGFP-C1. For MutH1-13, the deletion-mutants of tsAC were generated by PCR-based overlap extension. In this strategy, two pairs of primers were employed to get different part of tsAC. As the reverse primer of the first fragment was designed to complement to the forward primer of the second fragment, those two sequences could be ligated by overlap extension using only the forward primer of the first fragment and the reverse primer of the second. For MutH 8–10, a stretch of sequence without NLS was

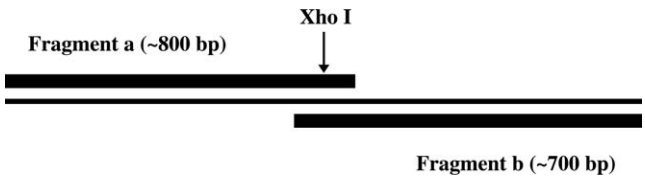

Fig. 1. Schematic view of the two fragments.
Fragment a and fragment b have a part of overlay, where there is an internal *Xho*I restriction site. The full-length tsAC could be obtained by restriction digesting and ligating by the *Xho*I site.