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Structural diversity of the cAMP-dependent protein kinase regulatory subunit in *Caenorhabditis elegans*

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ABSTRACT

The cAMP-dependent protein kinase (protein kinase A, PK-A) plays a key role in the control of eukaryotic cellular activity. The enzymology of PK-A in the free-living nematode, *Caenorhabditis elegans* is deceptively simple. Single genes encode the catalytic (C) subunit (*kin-1*), the regulatory (R) subunit (*kin-2*) and an A-kinase anchor protein (AKAP) (*aka-1*); nonetheless, PK-A is able to facilitate a comprehensive array of cAMP-mediated processes in this model multicellular organism. We have previously demonstrated that, in *C. elegans*, as many as 12 different isoforms of the C-subunit arise as a consequence of alternative splicing strategies. Here, we report the occurrence of transcripts encoding novel isoforms of the PK-A R-subunit in *C. elegans*. In place of exons 1 and 2, these transcripts include coding sequences from novel B or Q exons directly linked to exon 3, thereby generating isoforms with novel N-termini. R-subunits containing an exon B-encoded N-terminal polypeptide sequence were detected in extracts prepared from mixed populations of *C. elegans*. Of note is the observation that R-subunit isoforms containing exon B- or exon Q-encoded polypeptide sequences lack the dimerisation/docking domains conventionally seen in R-subunits. This means that they are unlikely to participate in the formation of tetrameric PK-A holoenzymes and, additionally, they are unlikely to interact with AKAP(s). It is therefore possible that, in *C. elegans*, in addition to tetrameric (R_2C_2) PK-A holoenzymes, there is also a sub-population of dimeric (RC) PK-A enzymes that are not tethered by AKAPs. Furthermore, inspection of the N-terminal sequence encoded by exon B suggests that this isoform is a likely target for N-myristoylation. Although unusual, a number of similarly N-myristoylatable R-subunits, from a range of different species, are present in the databases, suggesting that this may be a more generally observed feature of R-subunit structure. The occurrence of R-subunit isoforms, without dimerisation/docking domains (with or without N-myristoylatable N-termini) in other species would suggest that the control of PK-A activity may be more complex than hitherto thought.

1. Introduction

The cAMP-dependent protein kinase (protein kinase-A, PK-A) plays a fundamental role in cyclic AMP-mediated signalling processes in eukaryotes. In mammals, the PK-A holoenzyme is tetrameric in nature, consisting of two catalytic (C) and two regulatory (R) subunits [1]. Isoforms of the mammalian C subunit have been identified that are either the products of distinct genes (C_α, C_β, C_γ) [2], or arise as a consequence of alternative splicing strategies during transcript processing [3,4]. Additionally, multiple isoforms of the mammalian R-subunit have been identified (RIα, RIβ, RIIα and RIIβ) [5]. C_α, RIα and RIIα subunits are relatively abundant in a wide range of mammalian cells and tissues [6]. In contrast, C_β, RIβ and RIIβ subunits are more selectively expressed, being particularly abundant in brain tissues [7]. It is clear that, in mammals, expression of specialised R and C subunits has the potential to allow the generation of diverse PK-A holoenzymes across a range of highly differentiated cells and tissues.

In the nematode *Caenorhabditis elegans*, the PK-A C subunit is encoded by the *kin-1* gene [8] and the R-subunit is encoded by the *kin-2* gene [9]. There is now considerable evidence that the C subunit is expressed in multiple isoforms, arising as a consequence of alternative splicing events that lead to both N- and C-terminal variants of the C subunit [8,10]. Much less is known about the expression of R-subunit isoforms in *C. elegans*. Although the C-terminal region of the polypeptide, containing the cyclic AMP binding domains, shows significant similarity to mammalian R-subunits, the N-terminal region is much more divergent from the corresponding region in mammalian R-subunits. The only conserved areas within this region are the RII dimerisation/docking domain and a pseudosubstrate site characteristic of the mammalian RI family. It has been speculated that this

Abbreviations: PK-A, protein kinase-A (cyclic AMP-dependent protein kinase); C-subunit, catalytic subunit; R-subunit, regulatory subunit; RT, reverse transcriptase; EST, expressed sequence tag; AKAP, A-kinase anchor protein.

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N-terminal region may provide sufficient structural diversity to permit a single R-subunit to play multiple functional roles in the nematode [9].

The experiments described in the current paper were designed to investigate the occurrence and molecular basis of R-subunit diversity in *C. elegans*.

2. Materials and methods

2.1. Materials

Phusion Hot Start High Fidelity Polymerase was from Finnzymes. The pGEM-T Easy cloning vector was from Promega. The QIAprep Spin Miniprep kit, MiniElute gel extraction kit and MiniElute PCR purification kit were from Qiagen. The nucleic acid molecular mass markers, Hyperladder I and Hyperladder II, were from Bioline. Trizol, glycogen, oligo(dT)$_{20}$, Superscript III Reverse Transcriptase and primers for PCR were from Invitrogen. 8-AEA-cAMP-agarose [8-(2-aminoethylamino) adenosine-3′, 5′-cyclic monophosphate immobilised on agarose] was from Biolog. EDTA-free Protease Inhibitor Cocktail was from Roche. The pre-stained protein molecular mass markers were from Fermentas and New England Biolabs. Rabbit anti-human PK-A RIβ (C-19) (sc-907) was from Santa Cruz Biotechnology. Mouse anti-human PKA RIIα (612243) was from BD Transduction Laboratories. Peroxidase-conjugated anti-rabbit IgG and Pierce ECL Western Blotting Substrate were from Thermo Scientific. Sigma Water and ethidium bromide were from Sigma Aldrich.

Sequencing was carried out by the sequencing service at the University of Dundee, UK.

2.2. Animals

The Bristol N2 wild-type strain of *C. elegans*, from the MRC Laboratory of Molecular Biology, Cambridge, UK, was used in all experiments. Asynchronous cultures of *C. elegans* were maintained on *Escherichia coli* (NA22) essentially as described in [11].

2.3. Preparation of RNA

Total RNA, from mixed-stage nematodes, was isolated using a modification of the method described in [12]. Briefly, a 1:9 mixture of nematode suspension and Trizol was homogenised using a hand-held glass homogeniser. Chloroform was then added (same volume as the original nematode suspension) and the resulting mixture was centrifuged at 12,000×*g* for 15 min at 4 °C. The resulting aqueous phase, containing RNA, was transferred to a new tube containing 20 μg of glycogen: an equal volume of isopropanol was then added. This mixture was incubated at room temperature for 10 min and RNA was collected following centrifugation at 12,000×*g* for 10 min. RNA was washed with 75% (v/v) ethanol and then dissolved in water (Sigma).

2.4. Reverse transcriptase (RT)-PCR and semi-nested-RT-PCR

Transcripts encoding PK-A R-subunit were identified using semi-nested RT-PCR. Initially, single-stranded cDNA was synthesised from DNase-treated RNA, primed with oligo(dT)$_{20}$, using Superscript III Reverse Transcriptase at 50 °C for 50 min (total vol. 20 μl). Subsequently, 1 μl of the single-stranded cDNA preparation was added to Finnzymes' Phusion Hot Start High Fidelity PCR system buffer for PCR using appropriate sets of primers (total vol. 25 μl). Forward primers were based on unique sequences present in exons 1A, 1B, B and Q (see Fig. 1A and Table 1). Two sets of reverse primers were designed. The first set of reverse primers used for the first step of PCR corresponded to the 3′ ends of exons 3, 5 and 8 and the second set of reverse primers, for subsequent semi-nested PCR, was designed to the internal parts of exons 3, 5 and 8 (see Table 1).

The amplification mixture contained 0.5 μM of each primer (forward and reverse), 1 mM dNTP mixture, 1× PCR buffer (Bioline) and 1 U of Taq polymerase (Bioline). After initial denaturation at 94 °C for 1 min, PCR was performed for 30 cycles; denaturation, 94 °C, 30 s; annealing 65 °C (decreased 0.5 °C with each cycle down to 50 °C) for 30 s; extension 72 °C for 1 min and final extension, 72 °C for 5 min. RT-PCR products (10 μl) were subjected to electrophoresis on a 1.5% (w/v) agarose gel, stained with ethidium bromide and photographed on a UV transilluminator.

2.5. Subcloning and sequencing of RT-PCR products

Following fractionation of RT-PCR products on 1.5% (w/v) agarose gels, DNA was purified from appropriate ethidium bromide-stained bands using Qiagen MiniElute gel extraction and MiniElute PCR purification kits following the manufacturer's instructions. Purified DNA was cloned into pGEM-T Easy vector, under conditions specified by the supplier, and *E. coli* DH5α chemically competent cells were transformed. Transformed colonies were grown overnight at 37 °C. Plasmid DNA of positive clones was purified using a MiniPrep Qiagen kit. Plasmids containing the insert were sequenced.

2.6. Preparation of protein extracts

Nematodes were maintained in liquid cultures [12]. Harvested nematodes were stored at −80 °C until required. Nematodes (~2 g of nematodes obtained from a 50 ml culture) were thawed in the presence of an equal volume of PBS-TS buffer (50 mM KPO_4, 150 mM NaCl, 0.1% (v/v) Tween 20, 300 mM sucrose) containing protease inhibitor cocktail (1× Complete™ EDTA-free Cocktail tablet; Roche) [13]. The mixture was homogenised using a motorised glass homogeniser (2 ml of slurry was homogenised for a total of 4 min with 30 s breaks, and cooling on ice, after every minute). The resulting extract was centrifuged at 4 °C for 20 min at 20,000×*g*. The resulting supernatant was diluted with PBS-TS buffer to give a final protein concentration of ~3 mg/ml.

2.7. cAMP-affinity chromatography

The protocol used was an adaptation of that described in [13]. Cyclic AMP, immobilised on agarose (bed vol. 0.6 ml), was used to affinity purify cAMP-binding proteins. Following equilibration of the column with PBS-TS buffer, ~20–30 mg of protein (i.e. ~10 ml of 3 mg/ml extract prepared as described above) was loaded on to the column at ~50–200 μl/min. The column was washed with 6 column volumes of PBS-TS buffer to remove unbound protein. Subsequent washing, to remove non-specifically bound proteins, was with 1 column volume of 10 mM ADP in MiliQ water and 1 column volume of 5 mM cGMP in MiliQ water. Finally, cAMP binding proteins were eluted from the column with 2 column volumes of 100 mM cAMP followed by 4 column volumes of 8 M urea. Material from the eluant, and each washing step, was concentrated using a Millipore concentrator (cut-off ~5 kDa) to a final volume of ~30 μl and subjected to 1D-SDS-PAGE using 10% (w/v) acrylamide separating gels [14]. Following 1D SDS-PAGE, gels were subjected to Western blot analysis [15]. Electro-transfer of protein to PVDF membranes was carried out at 100 V for 1 h at 4 °C. Following incubation with the primary antibody and peroxidase-labelled secondary antibody, Western blots were developed using the Pierce ECL Western Blotting Substrate following the manufacturer's protocol.

2.8. Protein digestion

1D SDS-PAGE gels were stained with Coomassie Blue and the bands of interest were excised and subjected to digestion with trypsin or LysC. For this, the gel bands were de-stained in 100 μl of a 1:1 (v/v)