Gene 575 (2016) 429–437

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Identification and biochemical characterization of polyamine oxidases in amphioxus: Implications for emergence of vertebrate-specific spermine and acetylpolyamine oxidases

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ARTICLE INFO

Article history:
Received 12 March 2015
Received in revised form 6 August 2015
Accepted 8 September 2015
Available online 12 September 2015

Keywords:
Amphioxus
Branchiostoma
Polyamine oxidase
Spermine oxidase
Acetylpolyamine oxidase

ABSTRACT

Polyamine oxidases (PAOs) have been identified in a wide variety of animals, as well as in fungi and plant. Generally, plant PAOs oxidize spermine (Spm), spermidine (Spd) and their acetylated derivatives, N^1-acetylspermine (N^1-Aspm) and N^1-acetylspermidine (N^1-Aspd), while yeast PAOs oxidize Spm, N^1-Aspm and N^1-Aspd, but not Spd. By contrast, two different enzymes, namely spermine oxidase (SMO) and acetylpolyamine oxidase (APAO), specifically catalyze the oxidation of Spm and N^1-Aspm/N^1-Aspd, respectively. However, our knowledge on the biochemical and structural characterization of PAOs remains rather limited, and their evolutionary history is still enigmatic. In this study, two amphioxus (*Branchiostoma japonicum*) PAO genes, named *Bjpao1* and *Bjpao2*, were cloned and characterized. Both *Bjpao1* and *Bjpao2* displayed distinct tissue-specific expression patterns. Notably, rBjPAO1 oxidized both spermine and spermidine, but not N^1-acetylspermine, whereas rBjPAO2 oxidizes both spermidine and N^1-acetylspermine, but not spermine. To understand structure-function relationship, the enzymatic activities of mutant BjPAOs that were generated by site-directed mutagenesis and expressed in *E. coli* were examined, The results indicate that the residues H64, K301 and T460 in rBjPAO1, and H69, K315 and T467 in rBjPAO2 were all involved in substrate binding and enzyme catalytic activity to some extent. Based on our results and those of others, a model depicting the divergent evolution and functional specialization of vertebrate SMO and APAO genes is proposed.

1. Introduction

Polyamines (PAs), occurring most often in nature as spermine (Spm), spermidine (Spd) and putrescine (Put), are polybasic hydrocarbon chain molecules found in all cells across all kingdoms. PAs are involved in many biological processes, including cell growth, differentiation and apoptosis, via reversible electrostatic interaction with acidic molecules (e.g. DNA, RNA, nucleotides and proteins) through their strong polybasic character, affecting nucleic acid and protein synthesis, gene expression, protein function and regulation of ion channels (Tavladoraki et al., 2011; Polticelli et al., 2012). Maintenance of homeostasis of PAs in cells is necessary for these processes, but excess PAs can be toxic (Toninello et al., 2004; Amendola et al., 2009; Pegg, 2013), and thus have to be catabolized. The PA-catabolizing enzymes are known as polyamine oxidases (PAOs), which are flavin adenine dinucleotide (FAD)-containing enzymes capable of catalyzing the oxidation of PAs.

The substrate specificity of PAOs depends upon the source of the enzymes. Generally, plant PAOs oxidize Spm, Spd and their acetylated derivatives, N^1-Aspm and N^1-Aspd (Fincato et al., 2011), while yeast PAOs oxidize Spm, N^1-Aspm and N^1-Aspd, but not Spd (Landry and Sternglanz, 2003; Huang et al., 2005). Recently, the recombinant Pacific oyster PAO, the first one reported in invertebrates, is shown to be able to oxidize both Spm and N^1-Aspm, albeit with different efficiency (Cervelli et al., 2015). By contrast, two specialized PAO subfamilies, spermine oxidase (SMO; EC 1.5.3.16) and N^1-acetylpolyamine oxidase (APAO; EC 1.5.3.11) are identified in vertebrates (Polticelli et al., 2012). SMO catalyzes the oxidation of Spm, whereas APAO catalyzes the oxidation of N^1-Aspm and N^1-Aspd (Polticelli et al., 2012). Despite these enormous

Abbreviations: APAO, N^1-acetylpolyamine oxidase; DNA, deoxyribonucleic acid; DTT, dithiothreitol; FAD, flavin adenine dinucleotide; h, hour; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactoside; KDa, kilodalton(s); LB, Luria–Bertani (medium); M, mole per liter; MDL 72,527, N^1, N^4-bis (2, 3-butadienyl)-1, 4-butanediamine; Min, minute; N^1-Aspm, N^1-acetylspermine; N^1-Aspd, N^1-acetylspermidine; ORF, open reading frame; PAO, polyamine oxidase; PAGE, PA-gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pH, potential of hydrogen; Put, putrescine; pI, isoelectric point; qRT, PCR-quantitative real-time PCR; RACE, rapid-amplification of cDNA ends; RNA, ribonucleic acid; s, second; SMO, spermine oxidase; Spd, spermidine; Spm, spermine; SDS, sodium dodecyl sulfate; Tris, tris (hydroxymethyl) aminomethane.

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http://dx.doi.org/10.1016/j.gene.2015.09.017
0378-1119/

progresses, however, our knowledge on the biochemical and structural characterization of metazoan PAOs is still rather limited.

Members of PAO gene family have been identified from a variety of animals, including vertebrates, arthropodes, mollusks, nematodes and placozoa, as well as from fungi and plants (Polticelli et al., 2012). Notably, the presence of two related PAOs has been found in all the vertebrates and the cephalochordate amphioxus, but only a single copy of PAO gene been documented thus far in the invertebrates, including urochordates. Collectively, these suggest that a duplication event of an ancestral PAO-like gene resulted in the emergence of SMO and APAO in the vertebrates (Polticelli et al., 2012; Cervelli et al., 2015). However, the molecular process leading to the functional specialization of the ancestral gene remains largely elusive.

Amphioxus, a cephalochordate, has a vertebrate-like body plan including dorsal neural tube, notochord, segmented somites and pharyngeal gill slits, but it is less complex than vertebrates, having a genome uncomplicated by extensive genomic duplication (Putnam et al., 2008). It represents a close relative to vertebrates, which is the best available stand-in for the proximate invertebrate ancestor of vertebrates, and thus an ideal model organism for gaining insights into origin and evolution of vertebrates (Zhang et al., 2010). However, biochemical characterization of PAOs is still lacking in this evolutionarily important animal. The aims of this study are therefore to clone the PAO genes from the amphioxus *Branchiostoma japonicum*, named *Bjpao1* and *Bjpao2*, to analyze their expression patterns, and to examine their enzymatic activities and structure-function relation. This will certainly deepen our understanding of the molecular process leading to the emergence of SMO and APAO during vertebrate evolution.

2. Materials and methods

2.1. Cloning and sequencing of Bjpao cDNAs

Total RNAs were extracted with TRIzol (TaKaRa, Dalian, China) from adult *B. japonicum* collected during the breeding season (mid-June to mid-July) in the vicinity of Qingdao, China, and digested with RNase-free DNase (TaKaRa) to eliminate the genomic contamination. The first-strand cDNA was synthesized with reverse transcription system (TaKaRa) using oligo d(T) primer. To amplify the complete cDNA fragments of *Bjpao1*, polymerase chain reaction (PCR) was performed using the first-strand cDNA as template, in a volume of 20 μl PCR mixture containing 1× PCR buffer, 0.5 unit of Ex Taq DNA polymerase (TaKaRa) and 0.4 μM of the PAO gene-specific primers P1 and P2 (Table 1), which were designed on the basis of the putative PAO sequence found in the Florida amphioxus *B. floridae* genome database (http://genome.jgi-psf.org//Brafl1/Brafl1.home.html). PCR was carried out at 94 °C for 5 min, followed by 34 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min 30 s, and a final extension step at 72 °C for 7 min. To amplify the initial fragment of *Bjpao2*, the primers P17 and P18 (Table 1) were designed on the basis of the putative PAO sequence found in *B. floridae* genome database and PCR was performed as follows: 1 cycle of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s, followed by the final extension at 72 °C for 7 min. To get the full-length cDNA sequence, both 5′ RACE and 3′ RACE were performed using the gene-specific primers P19 and P21, and the gene-specific nested primers P20 and P22 (Table 1), respectively. The P19 and P21 as well as P20 and P22 were designed according to the initial region

Table 1
Sequences of the primers used in this study.

Primers	Sequence (5′-3′)	Sequence information
Primers for Bjpao1		
P1 (sense)	ATGACAACAGGGAGACCCCAGAT	Primer for *Bjpao1* cDNA
P2 (antisense)	TTACATTTTATACAAGTCTATAAGTCTAGAAGC	Primer for *Bjpao1* cDNA
P3 (sense)	TTCCAGCCTTCATTCCTCG	Primer for qRT-PCR
P4 (antisense)	CGGTGTTGGCGTACAGGTC	Primer for qRT-PCR
P5 (sense)	TTCCAGCCTTCATTCCTCG	Primer for qRT-PCR
P6 (antisense)	CGGTGTTGGCGTACAGGTC	Primer for qRT-PCR
P7 (sense)	CGGAATTCATGACAACAGGGAGACC	Primer for expression
P8 (antisense)	CCGCTCGAGTTACATTTTATACAAGTCT	Primer for expression
P9 (sense)	GCTC***CAA***GGCAACAAGGACAAC	Primer for mutation (H64Q)
P10 (sense)	GCTC***GAA***GGCAACAAGGACAAC	Primer for mutation (H64E)
P11(antisense)	CAGGTTGTCCCTAACTCAAGGTTGT	Primer for mutation (H64Q, H64E)
P12(sense)	GTTGAC***ATG***ATTTTTCTGAGATTTC	Primer for mutation (K301M)
P13(antisense)	AGTTCCGAAACCAAGACTACTTATT	Primer for mutation (K301M)
P14 (sense)	AACACCCTACATCACC***TAT***ACACAT	Primer for mutation (T460Y)
P15 (sense)	AACACCCTACATCACC***AGT***ACACAT	Primer for mutation (T460S)
P16(antisense)	ATAGTAGCCTCTCCTGCAAACAGC	Primer for mutation (T460Y, T460S)
Primers for Bjpao2		
P17 (sense)	GATGAGGTGAAGCCTGACGACT	Primer for *Bjpao2* initial region
P18 (antisense)	TGTGGTCGCCTTCAAGTTCAT	Primer for *Bjpao2* initial region
P19(antisense)	CAGAGGAGGCTGGAACAGAGTC	5′ RACE GSP PCR
P20 (antisense)	AGCAATACAGTCTGGAGGAATGG	5′ RACE GSP nested PCR
P21 (sense)	GAGGATGGGGTTTGGTGTAGTC	3′ RACE GSP PCR
P22 (sense)	GGACCAAGACCATAGCAACAGC	3′ RACE GSP nested PCR
P23 (sense)	TACATGGAAACGCTGAGTGAAGAGG	Primer for qRT-PCR
P24 (antisense)	TAAAAGAGTAGGAGCCGCAGGTC	Primer for qRT-PCR
P25 (sense)	CGGAATTCATGGCTTCCACGGGTCTG	Primer for expression
P26 (antisense)	CCGCTCGAGTCATGCTTACTAGCATACAGGTTA	Primer for expression
P27 (sense)	GATC***CAA***GGTACCATTGGGAAC	Primer for mutation (H69Q)
P28 (antisense)	CAGTTGGCACCAAACTCTAGAGTGTCTG	Primer for mutation (H69Q)
P29 (sense)	GATC***GAA***GGTACCATTGGGAACCC	Primer for mutation (H69E)
P30 (antisense)	CAGTTGGCACCAAACTCTAGAGTGTCTG	Primer for mutation (H69E)
P31 (sense)	GTCAAC***ATG***ATCTTCCTCACCTTCGAGC	Primer for mutation (K315M)
P32 (antisense)	TACACCAAACCCCATCCTCTCTATA	Primer for mutation (K315M)
P33 (sense)	CAGTGAGTTTTTCTCC***TAT***GTCCAT	Primer for mutation (T467Y)
P34 (antisense)	TGAGTGGCCTCCCCTGCAAACTGC	Primer for mutation (T467Y)
P35 (sense)	CAGTGAGTTTTTCTCC***AGT***GTCCAT	Primer for mutation (T467S)
P36 (antisense)	TGAGTGGCCTCCCCTGCAAACTGC	Primer for mutation (T467S)