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Three isozymes of peptidylarginine deiminase in the chicken: Molecular cloning, characterization, and tissue distribution

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ABSTRACT

Peptidylarginine deiminase (PAD; EC 3.5.3.15) is a post-translational modification enzyme that catalyzes the conversion of protein-bound arginine to citrulline (deimination) in a calcium ion dependent manner. Although *PADI* genes are widely conserved among vertebrates, their function in the chicken is poorly understood. Here, we cloned and sequenced three chicken *PADI* cDNAs and analyzed the expression of their proteins in various tissues. Immunoblotting analysis showed that chicken PAD1 and PAD3 were present in cells of several central neuron system tissues including the retina; the chicken PAD2 protein was not detected in any tissue. We expressed recombinant chicken PADs in insect cells and characterized their enzymatic properties. The chicken PAD1 and PAD3 recombinant proteins required calcium ions as an essential cofactor for their catalytic activity. The two recombinant proteins showed similar substrate specificities toward synthetic arginine derivatives. By contrast to them, chicken PAD2 did not show any activity. We found that one of the conserved active centers in mammalian PADs had been altered in chicken PAD2; we prepared a reverse mutant but we did not detect an activity. We conclude that chicken PAD1 and PAD3 might play specific roles in the nervous system, but that chicken PAD2 might not be functional under normal physiological conditions.

1. Introduction

Post-translational modifications of proteins are emerging as key processes in the regulation of many cellular functions. One such modification is protein deimination or citrullination, which is performed by peptidylarginine deiminases (PADs; EC 3.5.3.15), and involves the conversion of protein-bound arginine residues to citrulline residues in the presence of calcium ions (Rogers and Taylor, 1977; Sugawara, 1979; Takahara et al., 1983). This modification leads to a loss of charge on the protein that can affect its conformation and, consequently, alter its function and interaction with other proteins (Vossenaar et al., 2003; György et al., 2006; Horibata et al., 2012). Protein deimination appears to play an important role in keratinocyte (Méchin et al., 2007; Ying et al., 2009) and hair follicle differentiation (Tarcsa et al., 1997; Kizawa et al., 2011) in normal human skin, and to contribute to the epigenetic control of gene expression (Cuthbert et al., 2004; Wang et al., 2004; Denis et al., 2009) and apoptosis (Hung et al., 2007). Elevated levels of PAD activity and protein deimination have been found in rheumatoid arthritis (Schellekens et al., 1998; Girbal-Neuhauser et al., 1999; Yamada et al., 2005; Luban and Li, 2010; Wegner et al., 2010), and in human neurological diseases such as multiple sclerosis (Harauz and Musse, 2007; Moscarello et al., 2007), auto-immune encephalomyelitis (Raijmakers et al., 2006), Alzheimer's disease (Ishigami et al., 2005), amyotrophic lateral sclerosis (Chou et al., 1996) and glaucoma (Bhattacharya, 2009).

Five PADs, termed PAD1, PAD2, PAD3, PAD4 and PAD6, have been identified in mammals, and their corresponding genes have similar exon–intron maps clustered on a single chromosome regardless of species (Chavanas et al., 2004). A potential PAD similar to mammalian PAD2 exists in zebrafish (*Danio rerio*) (Ying et al., 2009) and rainbow trout (*Oncorhynchus mykiss*) (Rebl et al., 2010), and three PADs are present in fowl (*Gallus gallus*) (Ying et al., 2009), whereas homologues of PADs are absent in non-vertebrate organisms such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*.

It has been speculated that mammalian *PADI* genes arose by molecular evolution from a single vertebrate ancestral gene (Chavanas et al., 2004). Studying the properties and physiological functions of PADs from a wide range of animal species should lead to a better understanding of the fundamental function of deimination. However, few studies on PADs from animals other than mammals have been

Abbreviations: PAD, peptidylarginine deiminase; cPAD, chicken PAD; RT-PCR, reverse-transcription polymerase chain reaction; Tris, tris(hydroxymethyl) aminomethane; anti-chicken GAPDH, anti-chicken glyceraldehyde-3-phosphate dehydrogenase; *SDHB*, succinate dehydrogenase; *RCC2*, regulator of chromosome condensation 2; RACE, rapid amplification of cDNA ends; Ac-L-Arg, acetyl-L-arginine; Ac-L-Arg-*O*-Me, acetyl-L-arginine-*O*-methylester; Bz-L-Arg, benzoyl-L-arginine; Bz-L-Arg-*O*-NH_2, benzoyl-L-arginine-*O*-amide; Bz-L-Arg-*O*-Et, BAEE, benzoyl-L-arginine-*O*-ethylester; L-Arg-*O*-Me, L-arginine-*O*-methylester; Tos-L-Arg-*O*-Me, tosyl-L-arginine-*O*-methylester; CNS, central nervous system.

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carried out so far. Previously, we and others reported that PAD activity is present in the brain of chicken, frog, turtle and carp (Kubilus and Baden, 1985; Takahara et al., 1986b). The chicken genome database, which was established in 2004 (Consortium, I.C.G.S., 2004; Wallis et al., 2004), indicates that the chicken has three *PADI* genes that are homologues of mammalian *PADI*1, *PADI*2 and *PADI*3 (Ying et al., 2009). Thus, we designated these PADs as chicken PAD1 (*cPADI*1), PAD2 (*cPADI*2), and PAD3 (*cPADI*3), respectively.

In the present study, we found that the enzyme purified from the chicken brain was a homologue of mammalian PAD3. Recently, Lange et al. (2011) showed that deimination by chicken PAD3 is a developmentally-regulated modulator of secondary spinal cord injury. To date, however, the tissue distributions of chicken PADs, including PAD3, under physiological conditions have not been elucidated. Our understanding of PAD function would also be greatly improved if it could be determined whether the enzymatic properties of avian PADs are similar to those of the orthologous mammalian PADs.

The present study was initiated to elucidate the expression patterns of three chicken *PADI* genes and to determine their full-length cDNA sequences. We constructed an expression system using recombinant cPADs in insect cells and then characterized the properties of recombinant cPAD1 and cPAD3 enzymes. We found that cPAD1 and cPAD3 are specifically distributed in the brain, spinal cord and retina. By contrast, recombinant cPAD2 did not show enzyme activity. Collectively, our findings indicate that cPAD1 and cPAD3 play several roles related to neuron function in the chicken.

2. Material and methods

2.1. Experimental animals

Rhode Island Red chickens (*G. gallus*) were used in the present study (http://www.ansi.okstate.edu/breeds/poultry/chickens/rhodeislandred/index.htm). All procedures for animal management and surgery were performed in accordance with standard protocols of the Animal Care and Use Committee, Ibaraki University.

2.2. RNA isolation and semi-quantitative reverse-transcription PCR

RNAs were extracted from tissues of 1-day-old birds using TRIzol reagent (Invitrogen) and used to synthesize first-strand cDNAs. For each reaction, 2 μg of total RNA was mixed with SuperScript III reverse transcriptase (Invitrogen) and Oligo-d$(T)_{15}$ primer according to the protocols supplied by the manufacturer. Reverse transcription was performed for 60 min at 50 °C, and then the enzyme was inactivated by heating at 70 °C for 15 min. For semi-quantitative reverse-transcription PCR (RT-PCR), primer pairs for each *cPADI* were designed based on cDNA or chicken genome information: *cPADI*1, forward primer 5′-TGAGGGCCCTGATTTTGGCTATGTGACCC-3′ and reverse primer 5′-TGAGATATCCATGCTTGAGCAGGTCGCGGTTCC-3′; *cPADI*2, forward primer 5′-TGCAGAGAGCCTCTCTTCAAGG-3′ and reverse primer 5′-CGCAGTGCACCTCGCCGAGCCGCCCA-3′; *cPADI*3, forward primer 5′-GCTCAAGAAACACCAGAGAACAC-3′ and reverse primer 5′-ATTGCGGCCGCAACTGGCAGCTCAGGGCACCATCCGCCACCACTTGA AGG-3′; and *β-actin*, forward primer 5′-ATGGATGATGATATTGCTGCG-3′ and reverse primer 5′-AAGAAAGATGGCTGGAAGAGG-3′. PCR was performed with 5 μL of the first-strand cDNA, 200 mM of each dNTP, 0.5 μM of sense and anti-sense primers, and 1.25 units TaKaRa Ex Taq (TaKaRa). Typically, the PCR conditions were 45 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and elongation at 72 °C for 3 min, followed by final elongation at 72 °C for 5 min.

2.3. Cloning of cPADI1 and cPADI2 cDNAs

Based on chicken genome information (Ensembl ID: *cPADI*1; ENSGALG00000021714, *cPADI*2; ENSGALG00000000503), we obtained cDNAs harboring *cPADI*1 and *cPADI*2. In brief, nucleotide sequences were determined by amplification of the major regions of the cDNAs, including the 5′-terminal regions, using the first strand cDNAs synthesized with the oligo-d$(T)_{15}$ primer described above as templates. The following primer pairs were used for the amplifications: *cPADI*1, forward primer 5′-GGTCCACCCCGTCGTCATCGA-3′ and reverse primer 5′-AACCTCTATATCGGGATCCAGTGGCC-3′; and *cPADI*2, forward primer 5′-TGTGGGGGGTTGGTTGAGGAAG-3′ and reverse primer 5′-CTTCTACGTTCACTCCCTCTGTGT-3′. Each amplification was performed using 5 μL of single-stranded cDNA and 1.25 units TaKaRa Ex Taq (TaKaRa). To determine the nucleotide sequence of the 3′-terminal region of *cPADI*1, we amplified the first strand cDNA as a template with the primers 5′-ACCCGTGTGCCCCCTGGATG-3′ (forward) and 5′-CACTTTATTTTGAGATGCTCCAAATCCC-3′ (reverse). The remaining regions, including the 3′-terminal end of *cPADI*2, were amplified with 3′-Full RACE Core set (TaKaRa) and the gene specific primer 5′-ATCATGATGCAGCAGAATCAG-3′ according to the protocols supplied by the manufacturer.

2.4. cDNA cloning of cPADI3

We purified and determined internal amino acid sequences of a PAD from chicken brain and used this information to design primers to amplify *cPADI*3 cDNA. Purification of PADs from chicken brain was performed using a previously described method (Takahara et al., 1986a,b) and aminohexyl-Sepharose column chromatography according to the method of Kanno et al. (2000). Peptides derived from chicken PADs were separated by *in situ Staphylococcus aureus* V8 protease hydrolysis and SDS-polyacrylamide slab gel electrophoresis as described by Cleveland et al. (1977). Peptides of interest were electro-blotted onto a poly vinylidene difluoride membrane (Millipore) and subjected to amino acid sequence analysis using an automated peptide sequencer (Applied Biosystems, model 477A). First strand cDNA was synthesized using 4 μg of chicken brain poly$(A)^+$ RNA, by the above method, and random hexamer primers with Super Script™ II RNase H^- Reverse Transcriptase (Gibco-BRL). Reverse transcription was performed for 60 min at 37 °C, and the enzyme was then inactivated by heating at 90 °C for 5 min. Amplification was carried out using 5 μL of the first strand cDNA as the template and the primers 5′-(G/A/T/C)T(A/C/T)TTCTACGT(G/A/T/C)GA(G/A)GG-3′ and 5′-CTG(G/A/T/C)GGATGTC(G/A/T)AT(G/A/T)AT(G/A)TC-3′, corresponding the amino acid sequences IFYVEG and DIIDIPQL, respectively. The nucleotide sequences of the 3′- and 5′-terminal regions of the cDNAs were determined using 3′- and 5′-RACE methods (Frohman et al., 1988). In brief, 5.6 μg of chicken brain poly$(A)^+$ RNA was reverse-transcribed with the GAGA XhoI dT primer 5′-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTT-3′. The 3′-terminal region was amplified using the primer 5′-GACATCATCGACATCCCTCAGCT(G/A/T/C)TT-3′, corresponding to nucleotides 1732 to 1757 (Supplementary data Fig. S1C), as the forward primer and the XhoI GAGA primer 5′-AAAAACTCGAGACTAGTTCTCTCTC-3′ as the reverse primer. For the 5′-terminal region of the cDNAs, the first strand cDNA was synthesized with a reverse primer (complementary to nucleotides 355 to 374), and a poly(dA) tail was added to the cDNA with terminal deoxynucleotidyl transferase. Next, the 5′-terminal region of the cDNA was amplified with a NotI-d$(T)_{18}$ primer and another reverse primer (complementary to nucleotides 331 to 350). The amplified cDNA fragments were ligated into ddT-tail vectors prepared using pBluescript SK as described by Holton and Graham (1991), and nucleotide sequences were determined by sequencing each clone.

2.5. Construction of plasmids for expression in insect cells

To construct plasmids carrying recombinant PADs, we amplified the open reading frame of each *PADI* cDNA fused with an N-terminal 6× Histidine-tag sequence. *cPADI*1 was amplified as follows: forward primer, 5′-ACTGAATTCATGGGCCATCATCATCATCATCATAGCAGCGGCCATATCGAAGGTCGTATGTCCCAGCGCCAGGTCATTCAG-3′, with the underlined