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Identification of *Bombyx mori* 14-3-3 orthologs and the interactor Hsp60

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Abstract

The 14-3-3 protein family consists of evolutionarily conserved, acidic 30 kDa proteins composed of seven isoforms named β, γ, ε, ζ, η, θ, and σ in mammalian cells. The dimeric complex of 14-3-3 isoforms, acting as a molecular adaptor, plays a central role in regulation of neuronal function. Since aberrant expression of 14-3-3 is identified in the brains of Alzheimer disease and Parkinson disease, a convenient insect model, if it is available, is highly valuable for studying a pathological role of 14-3-3 in neurodegeneration. Here, we identified the silkworm *Bombyx mori* 14-3-3 orthologs, ζ and ε isoforms highly homologous in amino acid sequences to the human 14-3-3ζ and 14-3-3ε. By Western blot, the expression of ζ and ε isoforms was identified at substantial levels in the first instar larva, markedly upregulated in the second instar larva, and the highest levels were maintained in the late stage of larva, the pupa, and the adult. Furthermore, by protein overlay and immunoprecipitation, we identified Hsp60 as a 14-3-3-binding partner. The 14-3-3 proteins interacted with the N-terminal fragment of Hsp60. The 14-3-3ζ and ε isoforms, along with Hsp60, were expressed widely with overlapping distribution in larval and adult tissues, including brain, fat body, silk gland, Malpighian tube, midgut, ovary, testis, antenna, and pheromone gland. These observations suggest that a molecular adaptor 14-3-3 and a molecular chaperone Hsp60 cooperate to achieve a wide range of cellular functions in *B. mori*.

Keywords: *Bombyx mori*; Brain; 14-3-3 epsilon; 14-3-3 zeta; Hsp60

1. Introduction

The 14-3-3 protein family consists of acidic 30 kDa proteins conserved through evolution in eukaryotic cells. The family is composed of seven isoforms named β, γ, ε, ζ, η, θ, and σ in mammalian cells, 13 in *Arabidopsis*, and two in *Drosophila* (Ferl et al., 2002). A homodimeric or heterodimeric complex, composed of the same or distinct isoforms, constitutes a cup-like structure with a ligand-binding amphipathic groove (Fu et al., 2000). The dimeric complex acts as a molecular adaptor that mediates a phosphorylation-dependent interaction with key signaling molecules involved in cell differentiation, proliferation, transformation, and apoptosis (MacKintosh, 2004). More than 300 proteins, amounting to approximately 0.6% of the human proteome, have been identified as being 14-3-3-binding partners, distributed in all subcellular compartments (Jin et al., 2004; Meek et al., 2004; Pozuelo Rubio et al., 2004). Increasing evidence indicates that the 14-3-3 protein interacts with certain targets in a phosphorylation-independent manner (Borch et al., 2002; Dai and Murakami, 2003; Henriksson et al., 2002; Ottmann et al., 2007; Zhai et al., 2001). Although the high sequence conservation among distinct isoforms suggests functional redundancy, some degree of diversity argues for the isoform-specific biological role (Acevedo et al., 2007; Lau et al., 2006).

The 14-3-3 protein is expressed most abundantly in neurons in the human central nervous system (CNS), where it represents 1% of total cytosolic proteins (Boston et al., 1982; also see the review of Berg et al., 2002). Aberrant expression and impaired function

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Table 1
Primers utilized for PCR-based cloning

Gene name	GenBank accession number	Sense primers	Antisense primers	Cloning vector
14-3-3ζ isoform	AB378097	5′-tcttccatcgtccacgatgtccgt-3′	5’gagtgcggcgagatattagttgtc3’	p3T
14-3-3ζ isoform	AB378097	5′-tccgtcgacaaggaagaactggtg-3′	5’ttagttgtcgccgccctcggcagg3’	pTrcHis-TOPO
14-3-3ζ isoform	AB378097	5′-cggaattcctccgtcgacaaggaagaactg-3′	5’cgggatcccgttagttgtcgccgccctcgg3’	p3XFLAG-CMV7.1
14-3-3ε isoform	AB378098	5′-catttgtcatccacaatgtcggaaaggga-3′	5’aggggggaggcgcggccattacgagacgtc3’	p3T
14-3-3ε isoform	AB378098	5′-tcggaaagggaagataatgtgtat-3′	5’ttacgagacgtcctggtcctcgcc3’	pTrcHis-TOPO
14-3-3ε isoform	AB378098	5′-cggaattcctcggaaagggaagataatgtg-3′	5’cgggatcccgttacgagacgtcctggtcct3’	p3XFLAG-CMV7.1
Hsp60 N-terminal half (NTF; aa 2–261)	AADK01014978	5′-ttgcgtctacctcgtgttgttcgt-3′	5’tcacatttctaatgctggaatgat3’	pTrcHis-TOPO
Hsp60 C-terminal half (CTF; aa 262–572)	AADK01014978	5′-gctaatcaacagaggaagcctctg-3′	5’tcacatcatgcctcccataccacc3’	pTrcHis-TOPO
Hsp60 full-length (FL; aa 2–572)	AADK01014978	5′-ggggtacccctcacatcatgcctcccatac-3′	AADK01014978	pCMV-Myc

The PCR product was cloned into a cloning vector p3T for sequencing or into a prokaryotic expression vector pTrcHis-TOPO to produce a fusion protein with a Xpress tag in *E. coli*. It was also cloned into a mammalian expression vector p3XFLAG-CMV7.1 or pCMV-Myc to express a fusion protein with a flag tag or a Myc tag in HEK293 cells.

of 14-3-3 in the CNS are closely associated with pathogenetic mechanisms of Alzheimer disease, Parkinson disease, Creutzfeldt–Jacob disease (CJD), spinocerebellar ataxia, amyotrophic lateral sclerosis, and multiple sclerosis (Chen et al., 2003; Hsich et al., 1996; Kawamoto et al., 2002; Layfield et al., 1996; Malaspina et al., 2000; Satoh et al., 2004). Therefore, a convenient insect model, if it is available, is highly valuable for studying 14-3-3 function to clarify pathological mechanisms underlying human neurodegenerative and neuroinflammatory diseases.

Recently, the domesticated silkworm *Bombyx mori*, a Lepidoptera insect, has been utilized as a model system for basic science research because of its well-characterized genome, availability of various genetic mutants, and development of transgenic, RNAi and microarray technologies (Mita et al., 2004; Ohnishi et al., 2006; Tomita et al., 2003; Xia et al., 2004, 2007). The silkworm genome project, recently completed, indicated that the genome encodes approximately 18,510 genes, which include substantial numbers of mammalian orthologs (Mita et al., 2004; Xia et al., 2007). Furthermore, the large body size makes it a model suitable for studying brain development and function (Moto et al., 2003; Hossain et al., 2006).

In the present study, we have cloned the silkworm *B. mori* 14-3-3 orthologs, clarified the temporal and spatial expression pattern during development, and identified a 14-3-3 interactor. We identified two distinct 14-3-3 isoforms ζ and ε that are conserved through evolution from insects to mammals, and showed a widespread tissue distribution pattern. Furthermore, we found that the *B. mori* 14-3-3ζ and ε isoforms bind to the heat shock protein Hsp60. These observations put forth a possible scenario that 14-3-3, a molecular adaptor for signaling components, along with Hsp60, a prototype molecular chaperone works together to achieve a broad range of cellular functions in *B. mori*.

2. Materials and methods

2.1. The silkworm B. mori

The hybrid strain *Kinshu* × *Showa* was supplied from Ueda Sanshu Co., Nagano, Japan. They were reared on an artificial diet silkmate 2S (Nosanko, Tsukuba, Japan), and kept at 25 °C on a 12 h light/12 h dark daily cycle.

2.2. Molecular cloning of B. mori 14-3-3 orthologs

First, we searched the *B. mori* expressed sequence tag (EST) database on KAIKOBLAST (kaikoblast.dna.affrc.go.jp) by importing the *Drosophila melanogaster* 14-3-3ζ (NM_165742) or 14-3-3ε (NM_169796) sequence as a query, and identified representatives of *B. mori* 14-3-3 genes. To clarify the whole coding sequences, total RNA was extracted from the brain of the fifth instar larva on day 3 by using RNeasy mini kit (Qiagen, Valencia, CA, USA). DNase-treated total RNA was processed for cDNA synthesis using oligo(dT)$_{12–18}$ primers and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). cDNA was amplified by PCR using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) and the primer sets listed in Table 1. The amplified products were cloned into a cloning vector p3T (MoBiTec, Göttingen, Germany). The purified vectors were processed for sequencing by the dideoxynucleotide chain termination method on ABI PRIZM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

The open reading frame (ORF), deduced amino acid sequences, multiple alignment, phylogenetic tree, and homology search were investigated by computer-assisted programs, such as BLAST (blast.genome.jp), Genetyx ver 8.0 (Genetyx Co., Tokyo, Japan), and CLC Free Workbench ver 3.2 (CLC Bio, Aarhus, Denmark).

2.3. Recombinant proteins

The ORFs of *B. mori* 14-3-3ζ and 14-3-3ε, and the N-terminal half (NTF) spanning amino acid residues 2–261 of Hsp60 and the C-terminal half (CTF) spanning amino acid residues 262–572 of Hsp60 (GenBank accession no. AADK01014978), were amplified by PCR using PfuTurbo DNA polymerase and the primer sets listed in Table 1. They were cloned into a prokaryotic expression vector pTrcHis-TOPO (Invitrogen) and expressed in *E. coli* as a fusion protein with an N-terminal Xpress tag. Recombinant human interferon-stimulated protein ISG15 (ISG15) tagged with Xpress and β-galactosidase fragment (LacZ) tagged with Xpress were used as a negative control, as described previously (Satoh et al., 2004, 2005). Recombinant proteins were purified according to the methods described previously (Satoh et al., 2004, 2005).

2.4. Western blot analysis

To prepare total protein extract, the tissues of male *B. mori*, except for ovary, antenna, and pheromone gland derived from female, were carefully dissected under binocular microscope. They were homogenized in RIPA lysis buffer composed of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (Sigma, St. Louis, MO, USA), followed by centrifugation at 10,000 rpm for