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Identification of *Bombyx mori* Akt and its phosphorylation by bombyxin stimulation

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ABSTRACT

Akt, a Ser/Thr protein kinase involved in insulin signaling, was identified from the silkworm, *Bombyx mori*. *Bombyx* Akt (BomAkt) is composed of 493 amino acid residues including regions conserved in other Akts: the Pleckstrin homology and kinase domains, and a dual phosphorylation site essential for kinase activation. Commercially available antibodies against mammalian Akt and phosphoAkt were able to recognize BomAkt and phosphorylated BomAkt in HEK293 cells expressing BomAkt. Additionally, phosphorylation of BomAkt was detectable in insulin-like growth factor (IGF)-I stimulated-HEK293 cells expressing BomAkt. RT-PCR and immunoblotting analyses revealed that BomAkt is expressed ubiquitously in *Bombyx* larvae. Phosphorylation of BomAkt was observed both in the isolated fat body after exposure to bombyxin, an endogenous insulin-like peptide, and in the larval fat body by refeeding a diet after starvation. These results suggest that dietary intake may activate the insulin signaling pathway, including Akt, through bombyxin action in *B. mori*.

1. Introduction

Insulin-like peptides are essential for biological function in various organisms, not only vertebrates but also invertebrates (Wu and Brown, 2006). In vertebrates, insulin-like peptides exert a variety of bioactivities, such as induction of proliferation and differentiation, inhibition of apoptosis in a variety of cells, and regulation of metabolism. In general, the signaling pathway of insulin-like peptides, such as insulin and insulin-like growth factors (IGFs), requires binding of such ligands to their receptors activating receptor tyrosine kinases that phosphorylate several intracellular substrates including insulin receptor substrates (IRSs) (White, 2002). The phosphorylated substrates are recognized by the Src-homology (SH) 2 domain of other signaling molecules, including a p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase (Backer et al., 1992). The binding between IRS and p85 activates a catalytic subunit of PI 3-kinase (p110), and consequently produces phosphoinositide-3,4,5-triphosphate (PIP_3) (White, 2002). PIP_3 is a key signaling molecule that recruits proteins possessing the Pleckstrin homology (PH) domain such as Akt to the plasma membrane (Bluem-Jensen and Hunter, 2001). Akt is a Ser/Thr protein kinase, originally identified in mouse (Jones et al., 1991; Scheid and Woodgett, 2001). Akt is phosphorylated at the plasma membrane by PIP_3-activated phosphoinositide-dependent kinases (PDKs) at Thr^{308} and Ser^{473}, residues (human) that are conserved as a dual phosphorylation site (Meier and Hemmings, 1999). This phosphorylation of Akt is known to be necessary for full activation of the kinase (Nicholson and Anderson, 2002). Akt activation is involved in various bioactivities regulated by insulin-like peptides in vertebrates.

In invertebrates, the biological events modulated by insulin-like peptides have been extensively studied in a number of species. Abnormality in metabolism and the longevity of the nematode, *Caenorhabditis elegans,* is caused by a disorder in the insulin-like signaling molecules, DAF-2 (corresponding to the insulin receptor) and AGE-1 (corresponding to PI 3-kinase) (Hekimi et al., 1998). In the fruit fly, *Drosophila melanogaster*, dysfunction of the molecules in insulin signaling, including INR (corresponding to insulin receptor) (Tatar et al., 2003), Chico (corresponding to IRS) (Clancy et al., 2001) and PdIns-3-Ps (corresponding to PI 3-kinase) (Garofalo, 2002), results in biologically crucial phenotypes, such as morphological dwarfs and changes in the life span. Insect Akt is a Ser/Thr protein kinase that has been identified and characterized in three dipteran species, *D. melanogaster* (Franke et al., 1994; Andjelkovic et al., 1995), and two species of mosquitoes, *Anopheles gambiae*, and *Aedes aegypti* (Riehle and Brown, 2003). In *D. melanogaster*, it is reported that Akt regulates cell size, growth, proliferation (Scanga et al., 2000), and apoptosis (Liu and Lehemann, 2006; Staveley et al., 1998), possibly mediated by the somatic calorimetric control. In *A. gambiae*, oogenesis is strongly related to Akt activation in female ovary

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during the reproduction stage (Riehle and Brown, 2003). Consequently, Akt must be one of the important evolutionarily conserved molecules in the insulin signaling pathway among vertebrates and invertebrates.

In the silkworm *Bombyx mori*, a lepidopteran species, an insulin-like peptide, bombyxin, has been identified as a critical factor regulating insect molting and metamorphosis (Nagasawa et al., 1984; Mizoguchi, 1995). Increased glucose levels in the larval hemolymph stimulate bombyxin production in *Bombyx* larvae (Masumura et al., 1999). Also, lipid metabolism is regulated by bombyxin in *Bombyx* adults (Satake et al., 1999). Therefore, we speculate that bombyxin likely induces other biological activities that are recognized as insulin activities in vertebrates. In addition, a high level of bombyxin-like immunoreactivity was observed during the pupal stage, especially in female *Bombyx* (Mizoguchi, 1995), suggesting that bombyxin can also function as a factor controlling ovarian development in the female pupae (Fullbright et al., 1997). This function has also been demonstrated in mosquitoes (Riehle and Brown, 1999; Riehle and Brown, 2002). Therefore, the bombyxin signaling pathway in *B. mori* is considered to be similar to that of insulin-like peptides in vertebrates.

Although structures and functions of insulin-like peptides and their receptors in *B. mori* are closely related to those in vertebrates, the molecules involved in the signaling pathway remain to be elucidated. Little direct evidence has been demonstrated on whether a signaling molecule represented by Akt is activated in response to endogenous ligands or physiological status. In the present study, we identified and characterized the *B. mori* Akt by cDNA cloning. In addition, we have for the first time demonstrated that *Bombyx* Akt (BomAkt) is activated both by bombyxin treatment and by feeding diet.

2. Materials and methods

2.1. Chemicals and antibodies

Chemicals used in the present study were purchased from Kanto Chemicals (Tokyo, Japan). Synthetic bombyxin II was a kind gift from Dr. K. Nagata (Dep. of Applied Biological Chemistry, Grad. school of Agricultural and Life Sciences of the University of Tokyo). Anti-myc antibody was purchased from Upstate Biotech (VA, USA), Akt- and phospho-Akt-specific antibodies (S473) were from Cell Signaling Technology (MA, USA), and HA antibody was from Sigma-Aldrich (MO, USA).

2.2. Animals

Eggs of the silkworm, *Bombyx mori* (a hybrid strain, Kinshu×Showa), were purchased from a silkworm egg-producing company, Ueda Sanshu Ltd (Ueda, Japan). Larvae were reared in plastic containers at 25±2 °C with 70±5% relative humidity, and long-day lighting conditions (16 L:8D) as previously described (Nagata and Nagasawa, 2006). Larvae were fed daily with a fresh diet of Silkmate 2S artificial diet purchased from Nippon Nosan Co. Ltd. (Yokohama, Japan).

2.3. Molecular cloning of Akt from Bombyx mori

Several individuals of last instar day-2 *Bombyx* larvae were anesthetized on ice and the fat body was dissected out. Total RNA was extracted from the fat body using TRIzol® reagent (Invitrogen, CA) according to the manufacturer's protocol. The total RNA was treated with RQ DNaseI (Promega, WI, USA) and the resulting materials (approximately 1 μg) were reverse-transcribed for preparation of template cDNA using Superscript® III RNaseH-reverse transcriptase (Invitrogen, CA, USA) primed by an oligo (dT) primer, 5′-AAGGAGTGGTATCCAGTGTGCTGG$(T)_{30}$VN-3′. The partial cDNA sequence of the Akt homologue in the *Bombyx* EST-database, Silkbase; http://morus.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi, was confirmed by RT-PCR based on the obtained sequence using fat body-derived cDNA and a set of primers, 5′-TGTTTTA-TATTGGTGCTCTGCGTG-3′ and 5′-GAATTTTTTTAAGGTGATGCGGCG-3′, designed based on the sequences of brP-0794, ceN-1697, and NV02172 in Silkbase. PCR was performed using a GeneAmp® PCR System 9700 (Applied Biosystems, CA, USA) and TaKaRa Ex Taq polymerase (TaKaRaBio, Shiga, Japan) with the following PCR program; initial denaturation step at 94 °C for 1 min and 30 subsequent cycles of amplification (94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min). A cDNA fragment of the *Bombyx akt* homolog thus obtained was subcloned into pGEM-T easy vector (Promega, WI) and sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA) on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). 3′-RACE was performed using the primers 5′-GGCGGCGCCCGGGATCATCGTGAAGG-3′ and 5′-CCAGCACACTGGA-TACCACTCC-3′ (CD-SY), and the cDNA was amplified for 30 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s). For nested PCR, the primer set 5′-TCTCGGGTACCTCCACTCCGAGGG-3′ and CD-SY were utilized under identical PCR conditions. The entire cDNA covering the open reading frame (ORF) of BomAkt was amplified and sequenced using the following primers; 5′-GTGCTGGAAAGCAAACAGCTAGTGG-3′; 5′-GGGGCTAGTGCCGTTTGTCGGTCAG-3′. All sequencing analyses were carried out using partial fragments derived from the entire cDNA derived from at least 5 clones. Overlapped partial fragments from the entire cDNA were then sequenced from both 5′- and 3′-ends in every analyses to avoid misreading resulted from polymerase reaction in PCR steps. Tissue distribution analysis by RT-PCR was performed using primers for amplification of BomAkt ORF cDNA. The experimental control was performed under identical conditions of RT-PCR for *Bombyx* actin-1 cDNA using the primer set; 5'-TCTACAATGAGCTGCGTGTCGCCCCCGAGG-3′; 5′-ATTT-CCCTCTCAGCGGTGGTGGTGAACGAG-3′.

2.4. Plasmid construction

The ORF portion of BomAkt cDNA was amplified by PCR using *Bombyx* larval cDNA and the primers set; forward: 5′-GTGCTGGAAAG-CAAACAGCTAGTGG, reverse: GGGGCTAGTGCCGTTTGTCGGTCAG-3′. The amplified BomAkt cDNA was inserted into a pGEM-T easy vector (Promega, WI, USA) and the insertion was confirmed by DNA sequencing. Using the plasmid DNA harboring the BomAkt cDNA insert as a template DNA, an *akt* cDNA fragment was prepared by PCR using the following primers; forward: 5′-TTTTAGATCTAAGTGGC-GAGGCGCGAGGGCCATG-3′; reverse: SP6 primer. The cDNA fragment was digested with *Bgl*II and *Sal*I and inserted into *Bgl*II and *Sal*I sites of the mammalian expression vector, pCMV-myc (Sigma-Aldrich, MO, USA).

2.5. Expression of BomAkt in mammalian cells and immunoprecipitation followed by immunoblotting

HEK293 cells were maintained at 37 °C in a humidified CO_2-controlled atmosphere in DMEM supplemented with 10% calf serum, 0.1% $NaHCO_3$, 50 IU/mL penicillin, 50 μg/mL streptomycin, 0.5 μg/mL amphotericin B (Sankyo, Tokyo, Japan). The expression vectors were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen, CA, USA). Cells were serum-starved for 18 h followed by stimulation with IGF-I (100 ng/mL) for 2 min. Cell extract preparations and immunoprecipitation of cell extracts were performed as described previously (Hakuno et al., 2007). Briefly, cells were harvested in ice-cold lysis buffer [50 mM Tris–HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 100 KIU/mL aprotinin, 20 μg/mL phenylmethanesulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 5 μg/mL pepstatin]. The lysate was centrifuged at 14,000 ×*g* for 10 min at 4 °C. The amount of protein in the supernatant was estimated by BioRad Protein Assay Kit I (Bio-Rad,