

Evaluation of the Bombyxin Gene and Bombyxin Insulin-like Peptide in Silkworm (*Bombyx Mori*)

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Abstract: The aim of this study is to assess the insulin-like peptide bombyxin and some molecular aspects of silkworm (*Bombyx mori*). The interactions among bombyxin, bombyxin gene and amino acid sequences of bombyxin were verified. In this investigation, the miscellaneous dimensions of the bombyxin were reviewed.

Key words: *Bombyx mori*, insulin, peptide

INTRODUCTION

Peptides play a crucial role in many physiological processes including actions as neurotransmitters, hormones, and antibiotics. Research has shown their importance in such fields as neuroscience, immunology, pharmacology, and cell biology. A large number of neuropeptides has been identified in the brain of insects. At least 35 neuropeptide precursor genes have been characterized in *Drosophila melanogaster*, some of which encode multiple peptides. Additional neuropeptides have been found in other insect species. With a few notable exceptions, most of the neuropeptides have been demonstrated in brain interneurons of various types (Aizono, *et al.*, 1997a, Aizono, *et al.*, 1997b).

The products of each neuropeptide precursor seem to be co-expressed, and each precursor displays a unique neuronal distribution pattern. Commonly, each type of neuropeptide is localized to a relatively small number of neurons. Emphasis has been placed upon interneurons innervating specific brain areas, such as the optic lobes, accessory medulla, antennal lobes, central body, and mushroom bodies. The functional roles of some neuropeptides and their receptors have been investigated in *D. melanogaster* by molecular genetics techniques. In addition, behavioral and electrophysiological assays have addressed neuropeptide functions in the cockroach *Leucophaea maderae*. Thus, the involvement of brain neuropeptides in circadian clock function, olfactory processing, various aspects of feeding behavior, and learning and memory are highlighted in this review. Studies so far indicate that neuropeptides can play a multitude of functional roles in the brain and that even single neuropeptides are likely to be multifunctional (Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983). The excretory process of insects is controlled by both diuretic and antidiuretic hormones. In general, diuretic hormones act on Malpighian tubules to increase secretion of primary urine, whereas antidiuretic hormones stimulate fluid reabsorption in the hindgut (ileum ana rectum). There are, however, exceptions to this, with early reports of an antidiuretic factor acting on Malpighian tubules to reduce secretion of primary urine and of a diuretic factor that appears to decrease fluid uptake from the hindgut. The first identified peptide shown to reduce primary urine production was Manse-CAP2b, which is a cardioacceleratory peptide (CAP) from the tobacco hornworm, *Manduca sexta*. Manse-CAP2b acts via cyclic GMP to reduce secretion by serotonin-stimulated Malpighian tubules of the blood-sucking bug *Rhodnius prolixus*. Surprisingly, the same peptide acts via cyclic GMP and Ca²⁺ to stimulate primary urine production by Malpighian tubules of the fruit fly, *Drosophila melanogaster* (Aizono, *et al.*, 1997a, Aizono, *et al.*, 1997b; Dow and Davies, 2003; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Kondo, *et al.*, 1996; Terhzaz *et al.*, 2006). Subsequently, two other antidiuretic peptides (Tenmo-ADFa and Tenmo-ADFb) that act on Malpighian tubules have been identified from a pupal head extract of the mealworm beetle, *Tenebrio molitor*, based upon their ability to

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increase cyclic GMP production (Eigenheer *et al.*, 2002; Eigenheer *et al.*, 2003; Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983). Both peptides reduce secretion by the free (proximal) portion of Malpighian tubules from lastinstar mealworm larvae, an effect that is mimicked by exogenous cyclic GMP. Tenmo-ADFa is extraordinarily potent in the fluid secretion assay, with an EC50 of 10 fmol IB1 compared with 240 pmol IB1 for Tenmo-ADFb. Manse-CAP2b also stimulates cyclic GMP production by *T. molitor* tubules and reduces fluid secretion, but it is considerably less potent than either of the native peptides, with an EC50 of 85 nmol IB1 (Coast1, *et al.*, 2007; Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Kondo, *et al.*, 1996; Wiehart *et al.*, 2002).

Bombyxin:

Bombyxin is an insect neurohormone with an insulin-like structure. The N-terminal A chain helix, a region which is considered part of the active site in insulin, is almost identical between the two hormones. Bombyxin analogues with modifications at the N-terminus of the A-chain were synthesized and investigated for their ability to bind to bombyxin-specific receptors. While N-acetylation reduced the affinity to the bombyxin receptor to 18% the removal of glycine (A1) inactivated the hormone completely. Replacement of glycine (A1) by L-amino acids caused a significant loss in activity (11%) while its replacement by D-amino acid resulted in active bombyxin analogues (55%). Comparative CD spectroscopy indicated a change in structure for desGly(A1)bombyxin. Although the insect hormone does not have an insulin-like function it exhibits mammalian insulin-like structural sensitivity for A chain modifications (Aizono, *et al.*, 1997a, Aizono, *et al.*, 1997b; Bullesbach, 1999; Ebberink, *et al.*, 1989). Because invertebrates as well as vertebrates rely upon the same organic molecules for metabolism, both groups should, in theory, possess insulin. The experimental evidence in support of this notion comes from two different approaches: immunocytochemistry and biochemistry. With immunocytochemistry, rapid strides have been made in the identification of invertebrate cells and tissues that are reactive to anti-insulin. Most of the observations have been carried out with antisera raised to mammalian insulin, and positive results have been obtained in a range of different species, primarily insects and molluscs. In molluscs, immunoreactivity occurs not only in neuronal tissue, but also in the epithelia of the gut and hepato pancreas (Coast *et al.*, 2002a; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Kondo, *et al.*, 1996; Schooley *et al.*, 2005). Of course, there are problems and pitfalls in immunocytochemistry. The epitope for anti-insulin deduced from structure-activity analyses, is formed by the region including residues 8, 9, and 10 of the A-chain, and residues 2, 3, and 4 of the B-chain of insulin. The ability of invertebrate tissues to bind anti-mammalian insulin is surprising because non-mammalian insulins, insulin like growth factors, and relaxin are variable in this region and, therefore, do not bind antibodies to mammalian insulin. However, the neuroendocrine light green cells in the cerebral ganglia of the central nervous system of the freshwater snail have been identified as anti-porcine insulin immunopositive cells. Indeed, these cells produce an insulin-related peptide with a different epitope region. Bombyxin II consists of two non-identical chains: the A-chain of 20 residues, and the B-chain of 28 residues. Besides bombyxin II, two other peptides have been purified. Only the N-terminals of the A-chains of bombyxins I and III have been sequenced, and both have an 80% homology with bombyxin II. Four different forms of bombyxin-II have been published. A purification scheme consisting of 15 successive procedures for bombyxin from *Bombyx* heads has been established (Aizono, *et al.*, 1997a, Aizono, *et al.*, 1997b; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983). Bombyxin comprises many molecular forms which could be satisfactorily resolved only by high performance liquid chromatography (HPLC). Bombyxin-I, one of the heterogeneous molecular forms of bombyxin, was obtained as a single peak on an HPLC at the terminal step of purification, with a recovery of 8% and a purification fold of 2x10⁶. Fifty 119 of bombyxin. It has been obtained from 650,000 *Bombyx* heads and 0.1 ng of this pure material was able to evoke adult development when injected into a *Samia* brainless pupa (3x10¹¹ M in hemolymph). When incubated in vitro with a prothoracic gland taken from freshly ecdysed *Samia* pupa, bombyxin-I enhanced ecdysone release at a concentration of 1x10⁻¹¹ M. So far, other molecular forms named bombyxin- II, -III, -IV and -V have been purified to homogeneity. When the N-terminal 19 amino acid residues of bombyxin-I, -II, and III were determined, a surprising, unexpected fact was revealed: the sequences of bombyxins showed significant similarity with the N-terminal portion of the A-chain of insulin. At that time bombyxin was still called 4K-PTTH. When the complete sequence of bombyxin-II was determined, the similarity to insulin family peptides became even clearer. Bombyxin is a heterodimer consisting of two chains which some biologists named the A- and B-chains, and these chains are ~50% and ~30% similar to the A- and B-chains of human insulin, respectively (Fig. 1). Bombyxin resembles relaxin in having a pyroglutamic acid residue at the B-chain

N-terminus. So far, bombyxin-IV has also been sequenced fully and bombyxin-I, -III and -V have been partially sequenced. Two inter- and one intra-chain disulfide bonds are formed at the same positions as in insulin. Bombyxin-II and -IV have been chemically synthesized and the synthetic bombyxins showed the same biological activity as natural bombyxins. Molecular modeling for the three-dimensional structure of bombyxins has further shown that bombyxins resemble insulin in adopting the core structure similar to that of insulin. The presence of insulin-like molecules in insects had frequently been suggested by indirect evidence, but our finding of the amino acid sequence similarity between bombyxins and insulin was the first to demonstrate unequivocally the presence of insulin-related peptides in insects (Aizono, *et al.*, 1997a, Aizono, *et al.*, 1997b; Iwami, *et al.*, 1990; Iwami, 1995; Iwami, *et al.*, 1996a; Iwami, *et al.*, 1996b; Iwami, 2000; Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983). It is important that the structures of molluscan and insect insulins have been found at last, more than 50 years after the discovery of the first vertebrate insulin structure. The structure of the insulin molecule is largely determined by a characteristic arrangement of certain residues in its precursor. That similar arrangements of amino acids seen in the various insulins would have arisen independently in different branches of the phylogenetic tree is extremely improbable.

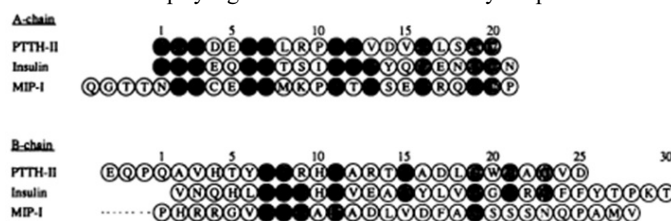


Fig. 1: Comparison of the amino acid sequences of the A and B chains of Bombyxin (PTTH) of *Bombyx mori*, human insulin and molluscan insulin related peptide (MIP) of *Lymnea stagnalis*. The amino acids are identified by their one-letter abbreviations.

The model depends critically on the relationship between such factors as the effects of sequence changes on the three-dimensional structure of the peptide, and the role of various parts of this structure in the conversion of the proinsulin to the active form, the storage of insulin, its transport to the site of action, and its interaction with the receptor (Ebberink, *et al.*, 1985; Ebberink, *et al.*, 1987; Ebberink, *et al.*, 1989; Emdin, *et al.*, 1985; Minnen, 1987; Nagasava, *et al.*, 1984; Nagasava, *et al.*, 1986). According to this hypothesis, the primary and three-dimensional structures conserved in vertebrate insulins must also be conserved in the related peptides of insects and molluscs. Since MIP, the vertebrate insulins and possibly bombyxin are involved in growth, it is important to discover whether the insulin receptors of invertebrates are homologous with those of vertebrates. According to currently accepted theory, the origin of insulin is to be found in the nervous systems of early multicellular organisms. Indeed, the localization of an invertebrate insulin within specific neurons of the brain would seem to support such a notion. Until now, the only insulin sequences available were from the central nervous systems of invertebrates and from the pancreas of vertebrate data that do not test the theory (Ebberink, *et al.*, 1989; Ebberink, *et al.*, 1985; Ebberink, *et al.*, 1987; Nagasava, *et al.*, 1984; Nagasava, *et al.*, 1986). The lobster cockroach *Nauphoeta cinerea* is an insect species well known for its male conspecific agonistic behavior. Two first-encountered adult males usually exhibit obvious agonistic behavior which includes kicking, biting, and chasing; while the subordinate retreats and stands still and flat. Depending on the population density, these encounters determine either in a high density or in a low density. The primary mediator of this agonistic behavior is the sex pheromone, which is only produced in the sternal glands of males and is composed of 3 major components: the genetically and developmentally correlated 2-methyl-thiazolidine and 4-ethyl-2-methoxyphenol, plus the genetically and developmentally independent 3-hydroxy-2-butanone. Later studies suggested that both cuticular hydrocarbons and sex pheromones are involved in establishing and maintaining social status (Aizono, *et al.*, 1997a, Aizono, *et al.*, 1997b; Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983; Chen, *et al.*, 2005). Neuroendocrine tissues and haemolymph from the tobacco hornworm, *Manduca sexta* L. and royal jelly from the honey bee, *Apis mellifera* L. contain insulin-like polypeptides. The hornworm peptide modulates trehalose levels when injected into the larval form of the same species while the honeybee factor also promotes glucose oxidation in vertebrate adipose tissue. Recently, evidence that a hypolipemic peptide from the locust, *Locusta migratoria*, and a lipogenic peptide from *Drosophila melanogaster* have some molecular features in common with mammalian insulin has been presented.

We report here the purification of insulin-like peptides from haemolymph of the tobacco hornworm and royal jelly of the honeybee (Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983; Kondo, *et al.*, 1996; Kramer, *et al.*, 1982). Peptide gonadotropins that regulate vitellogenesis and oogenesis in insects are generally considered to originate from the median neurosecretory cells (MNCs) of the brain, also known as pars intercerebralis, and to be liberated from the corpora cardiaca neurohemal complex. The major roles of these neurohormones include the stimulation of ovarian steroidogenesis, leading to the release of ecdysteroids into hemolymph. Indeed, beside their crucial function as molting hormones during immature stages, ecdysteroids also control several important aspects of reproduction in adult insects. In particular, they are directly involved in the regulation of vitellogenin biosynthesis in the fat body of higher Diptera. Although in other insects this important role is played by a sesquiterpenoid hormone (juvenile hormone), there is some evidence, at least in two species of locusts, that ecdysteroids still intervene and may be necessary for the onset of vitellogenesis. Until now, only two unrelated steroidogenic gonadotropins have been identified in two different insect species, ovarian maturing parsin (OMP) in *Locusta migratoria* and ovarian ecdysteroidogenic hormone (OEH) in *Aedes aegypti*. However, such neurohormones have not yet been isolated in flies. In particular, no putative homolog has been identified in *Drosophila*, despite the availability of the complete sequence of its genome. Nevertheless, our previous investigations in the blowfly *Phormia regina* have shown that at least two different brain factors are involved in the stimulation of ovarian steroidogenesis: the main one indeed originates from MNCs, whereas the other is elaborated in another, as yet unknown, part of the brain. Thus far, these factors have only been distinguished by their mode of action on ovarian steroidogenic cells, more particularly by their relationships with cAMP, the former acting via a cAMP-independent pathway, the latter via a cAMP-dependent one (Aizono, *et al.*, 1997a, Aizono, *et al.*, 1997b; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Kondo, *et al.*, 1996; Maniere, *et al.*, 2004). In the early 20th century, Japan's economy was highly dependent on silk production so a large amount of research was done on Bombyx. The abundance of the silkworm larvae was a great advantage for biologists at that time. In the course of the purification of PTTH, a peptide was identified in an extract from Bombyx brains and, after many years, the peptide was purified almost homogeneously from millions of Bombyx heads. The peptide has a molecular weight of 5 kDa and the ability to stimulate the prothoracic glands of the saturniid moth *Samia cynthia ricini* to synthesize and release ecdysone. Soon after, the 5 kDa peptide was however revealed to be inactive in Bombyx at a physiological dose, but instead a 30 kDa peptide is active in Bombyx itself. Thus, the 5 kDa peptide has not been regarded as a "pure" PTTH. Unexpectedly, the 5kDa peptide was shown to be homologous to insulin, a peptide hormone that plays crucial roles in the energy metabolism and growth of vertebrates. Although the existence of insulin-like peptides in invertebrates was predicted through biological and/or immunological assays, little was known about their structure. The elucidation of the structure of the 5 kDa peptide was therefore the first demonstration of the presence of insulin or insulin-related peptides in invertebrates at the molecular level. The silkworm insulin-related peptide is now called bombyxin and has been proved to exist widely in insects (Iwami, *et al.*, 1996a; Iwami, *et al.*, 1996b; Iwami, 2000; Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Kondo, *et al.*, 1996).

The Relationship Between Bombyxin and The Bombyxin Gene:

Bombyxin comprises highly heterogeneous molecular forms. Five molecular forms, I, II, III, IV and V, have so far been identified from Bombyx heads. The primary structures have been determined completely for bombyxins II and IV and partially for bombyxins I, III and V. They are heterodimers of the A and B chains whose amino acid sequences show about 50% and 30% identity to the A and B chains of human insulin (Fig. 2). The A and B chains of bombyxin are connected by two inter- and one intra-chain disulfide bonds in exactly the same manner as insulin. Bombyxins II and IV have been chemically synthesized and proved to have the same prothoracicotropic activity in *Samia* as natural bombyxins. Molecular modeling for the three-dimensional structure showed that bombyxin resembles insulin in adopting a globular-like core structure. Solution structure analysis of bombyxin by nuclear magnetic resonance further demonstrated that the overall main-chain fold of bombyxin is similar to those of insulin in solution, insulin in the crystalline T-state, and relaxin in the crystalline form. In fact, a hybrid molecule consisting of the A chain of Bombyx bombyxin and the B chain of human insulin stimulates 2- deoxyglucose uptake and DNA synthesis in CHO cells.

Bombyxin resembles relaxin in having a pyroglutamic acid residue at the B chain N terminus. thirty-two genes that encode bombyxin have been cloned from the Bombyx genome. These genes have been classified into 7 families, A, B, C, D, E, F and G, according to their sequence similarity. The family B members can

be further divided into three subfamilies BI, BII and BIII. The family A bombyxin consists of 10 gene copies, the family B 12 gene copies, the family C 6 gene copies. The bombyxin families D to G stand as single genes. The amino acid sequences deduced from the bombyxin genes show that bombyxin II is the product of genes A6 and/or A7 and bombyxin IV is that of gene E1. On the other hand, bombyxins III and V do not coincide with any bombyxins deduced from the genes, indicating that more bombyxin genes remain undetected. The deduced amino acid sequences of the bombyxin genes show 41% to 56% identity with each of the other families and 28% to 35% identity with human preproinsulin, as shown in Table 1. Preprobombyxins within the same family have at least 73% identical sequences with each other. Compared with the limited structural variation of vertebrate insulins, Bombyx bombyxin has a large diversity in structure. In vertebrate insulins, there may be little room for mutational divergence to occur because of the low copy number of their genes. It is reported that even point mutations resulting in abnormal human insulins can cause diabetes mellitus (Iwami, *et al.*, 1996a; Iwami, *et al.*, 1996b; Iwami, 2000; Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983; Kondo, *et al.*, 1996).

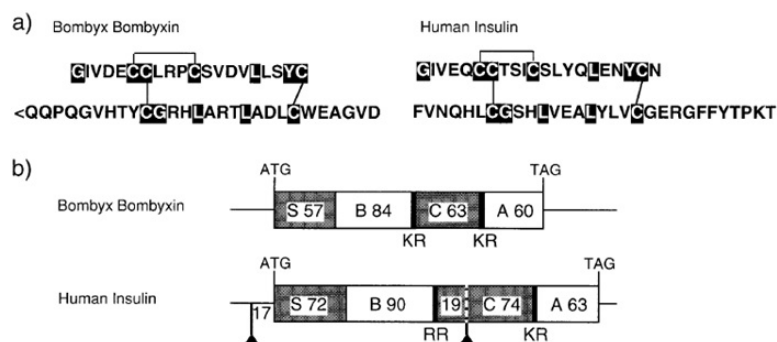


Figure 2. a) Amino acid sequences of Bombyxin II, one of the molecular forms of Bombyx bombyxin and human insulin. The identical residues between the two peptides are boxed. Disulfide bonds are indicated by bars. #Q pyroglutamate residue, b) Schematic representation of genes encoding prepropeptides for bombyxin (bombyxin A1) and human insulin. ATG and TAG represent the translation initiation and termination sites, respectively. Open boxes designate the domains that form mature molecules and hatched boxes those which are excised after translation. Dibasic amino acid residues, KR, RR, indicate the post-translation processing sites. S, signal peptide; B, B chain; A, A chain; C, C peptide. Numbers in boxes indicate the nucleotide number in the respective coding segments. Solid triangles indicate the positions of introns

Table 1: Overall sequence identity among the prepropeptides of Bombyx bombyxins and human insulin

		Bombyxin							Human Insulin
		A1	B1	C1	D1	E1	F1	G1	Human Insulin
Bombyxin	A1	(89)	100						
	B1	(73)	55	100					
	C1	(91)	51	51	100				
	D1		55	51	52	100			
	E1		48	49	47		100		
	F1		48	47	51	53	48	100	
	Gi		50	54	47	56	41	50	100
Human Insulin		35	33	34	33	34	31	28	100

The amino acid sequences of the protein products deduced from the genes are listed in Fig. 3. Except for C3 to C6, all the products have the same basic structure as that of preproinsulin. They consist of the signal peptide, B chain, C peptide, and A chain, in that order from the N terminus. Bombyxin genes C3 to C6 have an in-frame stop codon so as to encode only the signal peptide and an N terminal portion of the B chain. The bombyxin A10 gene also has an in-frame stop codon at about the center of the region coding for the A chain. These genes are thus presumably pseudogenes. The amino acid sequences of preprobombyxins show a high similarity in the A and B chains throughout the 7 families, and the level of conservation is remarkably high within each family (Fig. 3). On the other hand, the conservation of amino acid sequences is relatively low in the C peptide and even lower in the signal peptide, as has been shown for preproinsulins (Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983)

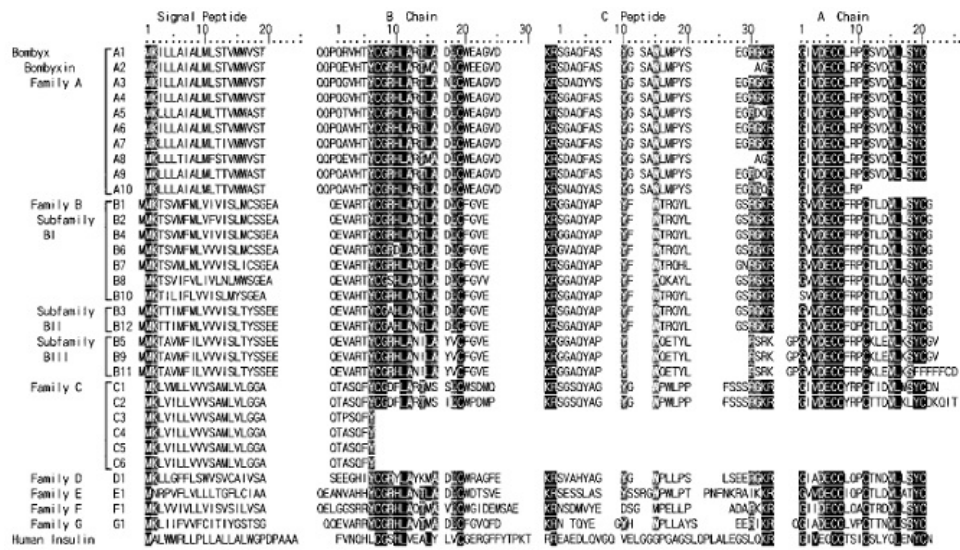


Fig. 3: Amino acid sequences of Bombyx preprobomoxins deduced from nucleotide sequences of their genes and human preproinsulin. Solid boxes represent identical residues among on insulin family prepropeptides listed and hatched boxes those among preprobomoxins. Amino acid residues are numbered from the N terminus of each domain of insulin.

The surface patch formed by the central part of the B chain is of critical importance for recognition by the bombyxin receptor. Intriguingly, the amino acid sequences of the surface patch are conserved or conservatively substituted among bombyxin members. Also conserved is glycine at position 8 of the B chain. In insulin, the glycine residue maintains the conformation through its contribution to the mainchain turn. The role of the N terminus of the bombyxin II A chain was investigated using bombyxin analogues with modifications. Modification of the A chain dramatically reduced the affinity to the bombyxin receptor, suggesting that bombyxin exhibits a mammalian insulin-like structural sensitivity for the A chain modifications. The paired basic amino acid residues are also conserved among preprobomoxins, which suggests that the mature bombyxins are generated through excision of the C peptide, exactly the same as insulin is. (Iwami, *et al.*, 1996a; Iwami, *et al.*, 1996b; Iwami, 2000; Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990).

Prothoracicotrophic hormone, an insect brain peptide, act on the prothoracic glands to stimulate the synthesis and release of ecdysone necessary for growth, moulting, and metamorphosis. The brain of the silkworm *Bombyx mori* contains, in addition to its own prothoracicotrophic hormone, a 5-kDa peptide bombyxin that manifests the prothoracicotrophic activity when tested with the saturniid moth *Samia cynthia ricini* and accordingly was referred to as 4K-prothoracicotrophic hormone previously. Bombyxin has been purified from *Bombyx* heads and shown to consist of highly heterogeneous molecular species. Bombyxins are heterodimers consisting of A- and B- chains whose amino acid sequence shows considerable homology with vertebrate insulin family peptides. The primary structure of bombyxin has been determined completely for three molecular species and partially for three others. These molecules differ from one another in a few replaced amino acid residues. The A- and B-chains are connected together through disulfide bonds in exactly the same way as in insulin. An immunohistochemical study using a monoclonal antibody raised against a synthetic fragment of bombyxin has revealed its localization in four pairs of mid-dorsal brain neurosecretory cells of *Bombyx*. Although the physiological function of bombyxin in *Bombyx* has remained unclarified, its insulin-like structure is thought to suggest some essential function in Bombyx growth or metabolism. The structure of preprobomoxin characterized here was shown to resemble that of preproinsulin in the domain organization of prepeptide/B-chain/C-peptide/A-chain the feature of which was also deduced from the analysis of genomic DNA containing a similar sequence. Thus, we presume the existence of a single-chain precursor molecule for bombyxin that assures the insulin-like tertiary structure and posttranslational processing to generate a mature bombyxin. Recently, a cDNA encoding the Amolluscan insulin-related peptide (MIP) of *Lymnaea stagnalis* has been characterized and the MIP gene has been shown to be expressed in the cerebral light-green cells that control the growth of *Lymnaea*. From these studies it is now unequivocally established that invertebrate nerve

cells produce peptides that share a common ancestral molecule with vertebrate insulin family peptides, the concept that had been suggested earlier mainly from immunological and biological studies and first been proved directly by primary structure determination of naturally occurring bombyxin. Amino acid sequencing of native bombyxin molecules revealed the presence of six different molecular species which differed from one another by replacement of a few amino acid residues and purification data suggested the presence of other bombyxin molecules yet to be sequenced. Furthermore, the bombyxin molecules deduced from the cDNA structure in the present study differed slightly in their sequence from those elucidated by peptide analysis. Thus, the presence of highly heterogeneous bombyxin molecules is now well established. However, it has not been known whether this heterogeneity results from the genetic variation associated with the use of millions of *Bombyx* heads for purification or is due to the presence of multiple bombyxin gene copies in the *Bombyx* genome. Southern hybridization studies reported here which revealed more than 10 copies of the bombyxin gene in the genomic DNA prepared from a single individual of *Bombyx* show that the latter is the case, although the former possibility might also hold true. In fact, our unpublished study on the cloning of bombyxin genes revealed the clustered localization of multiple bombyxin genes on a single DNA fragment of *Bombyx*. The presence of multiple bombyxin gene copies is in sharp contrast to vertebrate insulin genes which exist in a single copy except for the case of rat genes that have two copies. Four pairs of mid-dorsal brain neurosecretory cells of *Bombyx* have been immunohistochemically shown to contain bombyxin. Since XBb360-type bombyxin mRNA is contained in the fifth-instar 0-day *Bombyx* brain at a concentration of 4 pg or 1.1×10^7 molecules/Fg of brain total RNA and the four pairs of bombyxin-producing cells occupy $1/250$ volume of a *Bombyx* brain, the concentration of XBb360-type mRNA in these cells is calculated to be 2.8×10^9 molecules/pg of total RNA, based on the assumption that the total RNA is distributed evenly throughout the brain. This bombyxin mRNA concentration is comparable to the abundance of fibroin mRNA (1.3-5.1% (w/w) or $1.4-5.5 \times 10^9$ molecules / pg of total RNA in the posterior silk gland), which is synthesized in a remarkably high rate during a limited period of growth. The fibroin gene exists in a single copy and manifests a remarkably high transcriptional activity. In the case of bombyxin, it seems probable that the immense accumulation of mRNA is supported, at least in part, by multiplication of the gene (Adachis, *et al.*, 1989; Iwami, *et al.*, 1996a; Iwami, *et al.*, 1996b; Iwami, 2000; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Kondo, *et al.*, 1996). To date, many neuropeptide hormones such as prothoracicotropic hormone (PTTH), eclosion hormone and diapause hormone have been isolated from insects. PTTH is produced in two pairs of dorsolateral neurosecretory cells and released from the end of the axon at the corpus-allatum. The released PTTH stimulates the prothoracic gland to synthesize and secrete ecdysone. The change in ecdysone level results in the molt, and ecdysis is triggered by eclosion hormone. Diapause hormone released from the suboesophageal ganglion of a female pupa acts on the developing ovary to induce diapause eggs. *Bombyxin* is also a neuropeptide hormone with a molecular weight of 5 kDa that is produced in four pairs of mid-dorsal neurosecretory cells and secreted into the hemolymph. The hormone was originally isolated as a peptide from *Bombyx mori*, which showed prothoracicotropic activity to *Samia Cynthia ricini* but not to *Bombyx mori*. Accordingly, the physiological function of bombyxin in *Bombyx mori* has not been clarified as yet, although an important role of bombyxin in growth and development has been suggested based on its sequence homology to insulin, hypotrehalosemic activity and high titer during pupal-adult development. These neuropeptide hormones including bombyxin contribute to adaptation to environmental conditions by regulating development and morphological changes. Thus, the release of the neuropeptide hormones must be adjusted by environmental conditions such as light and temperature. However, the control mechanism of insect peptide hormones, for example when and how hormones are released, remains unknown (Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Kondo, *et al.*, 1996; Shirai, *et al.*, 2001). Immunostaining with anti-bombyxin antibody showed two of the eight bombyxin-producing neurosecretory cells in the mid-dorsal area (Fig. 4A). The rest of the bombyxin neurosecretory cells were detected on subsequential sections (data not shown). The diameter of these immunoreactive cells was 30-50 μ m. The antibody also stained axons on each bombyxin-neurosecretory cell extending to the median protocerebral area. The bombyxin-producing neurosecretory cells in the consecutive section were also immunostained with the anti-mAChR antibody (Fig. 3B). No immunoreaction was detected in the bombyxin-producing neurosecretory cells by normal rabbit serum (data not shown). These results indicate that bombyxin-producing neurosecretory cells express the mAChR. Interestingly, at higher magnification, intensely immunostained granules detected using anti-mAChR antibody were present just below the anterior plasma membrane and weak immunoreactivity against mAChR was observed throughout the cytoplasm of the bombyxin-producing neurosecretory cells. In that study, It has been unexpectedly found that several neurosecretory cells in the mid-dorsal area were

immunoreactive to the anti-mAChR, which resembled the bombyxin-producing neurosecretory cells. Accordingly, in the present study, we sought to verify whether the bombyxin-producing neurosecretory cells expressed mAChR (Iwami, *et al.*, 1996a; Iwami, *et al.*, 1996b; Iwami, 2000; Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Shirai, *et al.*, 2001).

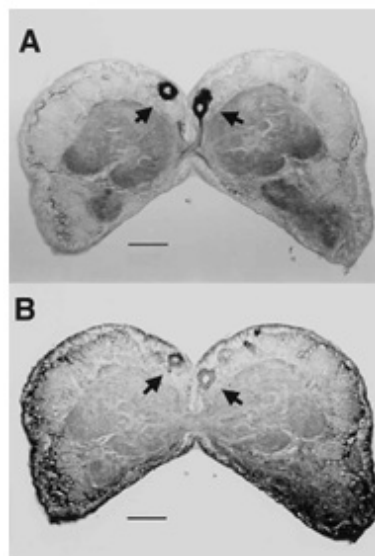


Fig. 4: Colocalization of bombyxin and mAChR in intercerebral neurosecretory cells. Two consecutive sections of brain were prepared as described in Materials and Methods. A: Arrows indicate two out of four pairs of bombyxin-producing neurosecretory cells detected by anti-bombyxin antibody. Axonal fibers from the bombyxin-producing neurosecretory cells were also stained in the median protocerebral area. B: AntimAChR antibody detected mAChR on the same neurosecretory cells shown in A. Scale bars are 100m m.

Bombyxins Belong to Insulin Family Peptides:

The most striking feature of bombyxins is the homology with insulin; the homology in A chain is approximately 50% and that of B chain about 40%. In addition, the mode of three disulfide bonds are completely identical with each other and the hydrophobic core residues in bombyxin-I1, A2 (Ile), Als (Leu), B14 (Leu) and B1a (Leu), are also identical with those of insulin. The glycine residues, which may contribute to the fold of peptide chains, at A, B11 and BZ6 in bombyxin-I1 is homologous with those of insulin. Based upon these facts, a three dimensional model of bombyxin-I1 has been constructed using computer graphics, showing that bombyxin-I1 can assume an insulin B like tertiary structure.

Recently the gene coding a bombyxin has been cloned using Bombyx gene library and synthetic DNA probe and the DNA sequence of the bombyxin gene indicates that bombyxins are biosynthesized in the form of prepropeptide followed by post-translational processing. Thus bombyxins can be considered to be a member of the insulin family peptides. In spite of considerable homology, insulin was inactivated the adult formation of brainless *Sarnia* pupa even at a dose of 1 μ g, 10⁴ times as much as the minimal active dose of bombs, and bombyxins failed to react with guinea pig antibody of porcine insulin. Bombyxins and insulin family peptides might have arisen from a common ancestral gene, which must be considerably conserved during the evolution to insects and mammals with different functions. From this view point, it is worthy of note that the presence of the gene coding an insulin-related peptide in Molluscan, termed molluscan insulin-related peptide (MIP) has recently been reported irrespective of the biological functions (Aizono, *et al.*, 1997a, Aizono, *et al.*, 1997b; Iwami, *et al.*, 1996a; Iwami, *et al.*, 1996b; Iwami, 2000; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Kondo, *et al.*, 1996; Suzuki, *et al.*, 1989).

Bombyxin was first identified as a neurosecretory hormone in the silk moth, *Bombyx mori*. Bombyxin has been isolated because of its ability to stimulate ecdysone secretion by the prothoracic glands of the moth *Samia cynthia* and was therefore believed to be the long-sought prothoracicotrophic hormone of insects. Unfortunately, bombyxin was subsequently shown not to stimulate ecdysone secretion in *B. mori* itself and does not appear to be the natural prothoracicotropin. The actual prothoracicotrophic hormone has since been identified and

clearly has no structural relationship to bombyxin. Bombyxin is a member of the insulin family of peptides, and its structure and molecular biology have been extensively studied. Because of bombyxin's similarity to insulin, its possible role in carbohydrate metabolism has been studied. The similarity of bombyxin to insulin and insulin-like growth factors has also led some authors to suggest that bombyxin may play a role in the regulation of growth. The work reported below shows that bombyxin functions as a growth factor for wing imaginal disks. Many of the protein components of hemolymph in insects are synthesized by the fat body, and conditioning of the culture medium by culturing fat body for 24 h often enhances the growth of cultured insect cells. In *Drosophila melanogaster*, the fat body is known to produce a growth factor for imaginal disks cells. Hence we investigated whether the fat body of *Precis* was the source for our putative growth factor. Conditioning the culture medium by culturing fat body for 24 h, followed by culture of imaginal disks (with or without the continued presence of fat body), did not support the growth of imaginal disks, even in the presence of an optimal concentration of 20-hydroxyecdysone. However, adding homogenates of brains of *P. coenia* (two brain-equivalents per 300 μ l of medium) strongly stimulated wing imaginal disk growth in the presence of ecdysone. The same result was obtained with brain homogenates and homogenates of subesophageal ganglia of *Manduca*, but not with homogenates of subesophageal ganglia of *Precis*. Evidently the active factor is not a general component of nervous tissue, because homogenates of subesophageal ganglia of *Precis* were inactive. These results show that the putative growth factor is not species-specific, because *Manduca* brains can stimulate normal growth in *Precis* wing imaginal disks, and that species may differ in the source(s) of this factor. Of the insect neurosecretory hormones, the one most likely to act as a growth factor is the hormone bombyxin, because bombyxin is a member of the insulin family, and other members of this family play a critical role in the control of growth in *Drosophila*. The neurohormone bombyxin, together with the steroid hormone 20-hydroxyecdysone, is required for normal growth of wing imaginal disks of the butterfly *P. coenia*. Bombyxins are insulin-like proteins with molecular masses between 4,500 and 5,000 Da. Like insulin, bombyxin is a heterodimer, with A and B chains connected by disulfide bonds. In *B. mori*, there are at least 38 bombyxin genes (a few of which are pseudogenes), and these are classified into five families (named ABE), based on sequence similarities. Bombyxin genes have also been identified in *Samia cynthia* (Saturniidae), which has six genes, and *Agrius singulatus* (Sphingidae), which appears to have three genes. In both the latter species the bombyxin genes belong to the same two families, related to the A and B families of *B. mori*. Several of the bombyxins are neurosecretory products, although the locations of expression of most of the bombyxins have not been determined. Not only are insulin-like proteins required for the growth of wing imaginal disks, but the insulin receptor signaling pathway is also a key regulator of overall growth and body size in *Drosophila* (21, 30-36). Overexpression of one of the *Drosophila* insulin genes causes an increase in organ and body size caused by an increase in cell size and cell number. By contrast, partial loss-of-function mutations in the insulin receptor, as well as in other molecules of the insulin signaling pathway, cause an increase in development time and a severe, but proportional, reduction in body size. Localized overexpression of the insulin receptor leads to localized cell proliferation and overgrowth of cells. It has been noted that mutational deficiencies in the insulin signaling pathway cause a reduction in body size that is similar to that obtained by starvation or by growth on a nutrient-deficient diet, suggesting that the insulin pathway is involved in mediating the growth response to variation in nutrition. It appears that the growth of imaginal tissues does not respond directly to the level of nutrients in the hemolymph. Instead, the level of nutrients is sensed by the brain or the fat body, depending on the species, and this organ regulates the growth of internal tissues through the secretion of growth factors (Iwami, *et al.*, 1996a; Iwami, *et al.*, 1996b; Iwami, 2000; Kawakami, *et al.*, 1989; Kawakami, *et al.*, 1990; Kondo, *et al.*, 1996; Nijhout, *et al.*, 2002).

Bombyxin is a 5 kDa secretory brain peptide that belongs to the insulin family. Bombyxin of the silkworm *Bombyx mori* can induce adult development when injected into brain-removed dormant pupae of the saturniid moth *Samia cynthia ricini* by activating the prothoracic glands to synthesize and release ecdysone. Bombyx bombyxin has been shown to lower the concentration of the major haemolymph sugar, trehalose, and to elevate the trehalase activity in the midgut and muscles in *Bombyx*, but the doses required to be effective are higher than the amounts in the feeding larvae. The exact physiological function of bombyxin in *Bombyx* itself is therefore still obscure, but its insulin-like structure suggests it has important roles. Bombyxin comprises a mixture of highly heterogeneous molecular forms whose amino acid sequences have 40% identity with human insulin. The *Bombyx* bombyxin gene encodes a precursor consisting of the signal peptide, B chain, C peptide, and A chain, in that order from the N terminus. So far, 32 bombyxin genes have been identified in *Bombyx*, and they are classified into 7 families, A to G, according to their sequence similarity. The bombyxin genes have no introns and cluster in unique distribution patterns. The gene arrangement in the cluster has been

classified into three categories: gene pairs, gene triplets, and single genes. Nucleotide sequence analysis indicates that equal and unequal crossings-over and duplications may have generated these unique distribution patterns. The *Bombyx bombyxin* genes are expressed predominantly in the brain and at low levels in a number of other tissues. Genes of all 7 families are expressed in four pairs of the medial neurosecretory cells of the brain. Detailed examination indicated that only a limited number of genes in the A, B and C family members are expressed and that their expression shows a gene-arrangement-dependent pattern (Ishizaki, *et al.*, 1983; Iwami, *et al.*, 1989; Iwami, 1990; Iwami, *et al.*, 1990; Iwami, 1995; Iwami, *et al.*, 1996a; Iwami, *et al.*, 1996b; Iwami, 2000; Hironori, *et al.*, 1994; Ebberink, *et al.*, 1989; Ishizaki, *et al.*, 1983).

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