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Cloning and Characterization of Ribosome-associated Membrane Protein 4 (RAMP4) gene in silkworm *Bombyx mori*

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Ribosome-associated membrane protein 4 (RAMP4) is a membrane protein that exposes its N-terminal hydrophilic portion on the cytoplasmic side and spans the membrane close to the C-terminal end. RAMP4 has previously been reported to belong to the set of proteins that remains associated with membrane-bound ribosomes, and controls the glycosylation of major histocompatbility complex class II-associated invariant chain. RAMP4 also may be relative to the stabilization of membrane proteins in response to stress, with other components of translocon, and molecular chaperons in ER. Application of 5'-RACE technique with specially designed primer, we cloned a 715 bp cDNA fragment which contains a 195 bp ORF, termed RAMP4. The deduced protein has 64 amino acid residues and contains a putative transmembrane-spanning domain at the COOH terminus.

Key words: RAMP4, Ribosome-associated membrane protein 4, *Bombyx mori*, RACE

Introduction

Baculoviridae is a family of enveloped, double-stranded DNA viruses that infect arthropods. *Bombyx mori* nuclear polyhedrosis virus (BmNPV) was the first virus discovered in the past studies of insect virology (Lu, 1998). Although the systemic infection process in vitro of BmNPV has just recently been reported (Rahman, 2004), little about the molecular mechanism in the NPV infection pathways in vivo has been clarified so far by now.

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In our former research, we applicated the fluorescent differential display (FDD) technique to analyzed the differential expression of genes related to highly susceptible silkworm strain 306 between exposure of BmNPV and not. We found a cDNA fragment from *Bombyx mori* which has high homology to *Bombyx mori* EST (GenBank access in number: AU235175). Using 5'-RACE, we cloned a 715 bp cDNA containing a 195bp open reading frame (ORF). Blasted the deduced amino acid sequence in Genbank, the suggested protein was found to be identical to ribosome-associated membrane protein 4 (RAMP4).

RAMP4 is a membrane protein that exposes its N-terminal hydrophilic portion on the cytoplasmic side and spans the membrane close to the C-terminal end (Schroder *et al.*, 1999). Previous study showed that RAMP4 belonged to a set of proteins that remains associated with membrane-bound ribosomes upon solubilization with the mild detergent digitonin (Görlich and Rapoport, 1993). The orientation of RAMP4 in the ER is likely to present the NH2 terminus to the cytosol, as predicted by previous studies of other similar proteins (Hartmann *et al.*, 1989; Kutay *et al.*, 1993), and a putative transmembrane-spanning domain at the COOH terminus probably anchors RAMP4 in the ER membrane (Yamaguchi *et al.*, 1999). RAMP4 was originally copurified with the core component of the protein-translocation machinery of the ER, the Sec61 complex, and it was recently reported that RAMP4 controls the glycosylation of major histocompatbility complex class II-associated invariant chain (Schroder *et al.*, 1999). While cells subjected to ER stress, RAMP4 overexpressed, and aggregated or degradated the integral membrane proteins, RAGE and CD8, members of the Ig superfamily of cell surface molecules with a single transmembrane-spanning domain, and facilitated glycosylation after the stress (Yamaguchi *et al.*, 1999). SERP1/RAMP4 can also be coprecipitated with calnexin (Yamaguchi *et al.*, 1999). Calnexin is a

membrane protein and a molecular chaperon in ER known to associate with folding intermediates of glycoproteins (monomeric glycoproteins) and believed to play a major role for the quality control apparatus in ER (Wada *et al.*, 1997). Collaborating with components of the translocon (such as Sec61α and Sec61β), and ER chaperons (such as calnexin), SERP1/RAMP4 is related to the stabilization of membrane proteins during and after ER stress (Yamaguchi *et al.*, 1999).

Many RAMP4 genes of different organisms had been reported in GenBank. Most of these genes belonged to vertebrata, while only a few belonged to insecta. This is the first case of ribosome-associated membrane protein 4 (RAMP4) gene reported in *Bombyx mori*, even in lepidopteran insect. Alignment of the amino acid sequence of RAMP4 from 15 organisms was performed and the gene sequence was analyzed by bioformatics, which may provide information for the further investigation.

Materials and Methods

Materials

The silkworm *Bombyx mori* was inbred in our lab. Highly susceptible silkworm strain 306 were used for this study. All larvae were raised to the fifth instars. The larvaes of newly metamorphosed 5th instar were fed with mulberry leaf that treated with 2×10^7 BmNPV. About 50 silkworms were collected to put up a RNA pool. Rneasy Mini Kit was purchased from QIAGEN, and BD SMART RACE cDNA Amplification Kit was from BD Bioscience Clontech. PCR reagents and PM18-T vector were obtained from Takara Company (Dalian). Other reagents were purchased from Shanghai Sangon Bio-technology Corporation.

RNA extraction

The midgut was dissected from the larvae at the 3rd day of the 5th instar, frozen with liquid nitrogen and ground into powder. Total RNA was extracted used the Rneasy Mini Kit according to the user manual. Finally, the total RNA inspected with Gene spec III (Naka Instruments Co., Ltd.) and stored at -70°C for further use.

RT-PCR and 5'-RACE

We used 1 μg total RNA as a template in the first-strand cDNA synthesis. And the specific primer-5'-TGAACTG-GATTTCCCTGGTCATC-3'-was designed to 5'-RACE based on the known sequence of our former research. The primer used to perform RACE was designed in the website (http://www.genefisher.de/). PCR reaction was carried out for 35 amplification cycles (94°C /30 sec, 62°C /30 sec, 72°C /3 min) in a Gene Amp 2700 System thermocycler. PCR products were examined by electrophoresis in 1% agarose gel with the thidium bromide staining.

Cloning and sequencing

The specific fragment was ligated into PM18-T vector and then transformed *E. coli* (DH5á strain). Plasmid was purified with MiniBEST Plasmid Purification Kit (Takara). The sequencing was performed using an automatic sequencer: CEQ8000 (Beckman Company).

Genomic analysis by bioinformatics

In order to establish the DNA sequence of this cDNA, the cDNA sequence was blasted to the contigs of *bymbox mori* Genomes in the National Centre for Biotechnology Information (NCBI) internet site (www.ncbi.nih.nim.gov). SIM4 (http://pbil.univ-lyon1.fr/sim4.php) (Florea *et al.*, 1998) was used to align the cDNA sequence with the genomic sequences to search the introns. We also used Splice Site Prediction of Neural Network (NNSSP) (http://www.fruitfly.org/seq_tools/splice.html) (Reese *et al.*, 1997) to predict the potential Splice sites.

Promoter and poly-A signals prediction

Neural Network Promoter Prediction (NNPP) (http://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001) was used to generate putative promoter elements. To predict the TATA-boxes, Hamming-Clustering Method for TATA Signal Prediction in Eukaryotic Genes (Hctata) (http://l25.itba.mi.cnr.it/~webgene/wwwHC_tata.html) (Milanesi *et al.*, 1996) and TATA-box prediction tool (http://wwwmgs.bionet.nsc.ru/mgs/programs/bdna/tata_bdna.html) were used. PolyA Scan (http://www.gene-regulation.com/cgi-bin/pub/programs/polyascan/polyascan.cgi) and Hamming Clustering poly-A prediction in Eukaryotic Genes (Hcpolya) (http://l25.itba.mi.cnr.it/~webgene/wwwHC_polya.html) (Milanesi *et al.*, 1996) were used to search for polyA signals through the whole DNA sequence.

Amino acid sequence prediction and analysis

We used the ExPASy Translate tool (http://au.expasy.org/tools/dna.html) to deduce the cDNA into amino acid sequence, and homologous analysis were performed using the Blast tool in GenBank (Blastx) and SIB BLAST Network Service (http://au.expasy.org/tools/blast/). Transmembrane regions in the deduced amino acid sequence were predicted by SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html). And SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen *et al.*, 2004) was used to predict the signal peptide cleavage sites of this amino acid sequence.

Results and Discussion

cDNA sequence

We sequenced a clone with a 715 bp fragment inserted, which contains a 195 bp ORF, having a potential to edcode a peptide of 64 amino acid residues. The initiating code ATG and the stopping code TAA (Fig. 1) are at the positions of 98 and 290, respectively.

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                                                ACTTTTATCTACGTGTCAAGCGAAATTGCGCGTTGTG  -61
GCTCTTCGAGTCCTTTTAATTTATTATTAAACTGACAAATATACTTTATAACAAAACAAA  -1
ATGGCCCCTAAGCAGAGAATGCGTATCGCCAACGAGATCGCCAGTAAAAACATCACAATG  60
M  A  P  K  Q  R  M  R  I  A  N  E  I  A  S  K  N  I  T  M   20
AGGGGGAATGTACCCAAAACTACTAAGGAAAAAGAAGACCAATATCCTGTGGCACCCTGG  120
R  G  N  V  P  K  T  T  K  E  K  E  D  Q  Y  P  V  A  P  W   40
CTCCTTGCTCTCTTCATCTTCGTAGTGTGTGGCTCTGCTGTGTTCCAGATAATCCAATCA  180
L  L  A  L  F  I  F  V  V  C  G  S  A  V  F  Q  I  I  Q  S   60
ATAAGACTAGCTTAAATCACGAAGACAACTGGAGAACTGCTATCGAAAACACAATAAAAT  240
I  R  L  A  *                                                  64
AACAACACTATCCATCTAAAGTTAAGGCACAATTTACACCAGCATCTCTTCATCGACATG  300
AGAGAGAGTGTTTTCGGAGGCGGCCATTAGTACCTTTAACTGTGCAATCGTGGCTTGATG  360
TCTCTTTCACACTTACAGGGTTAAAACGGGACGCCGATACATAAGGTTATTAGGGACGTT  420
GAGGTATCGGTTGTAGAATTTTCGATGAATTTTTGTCTCTTTTTCAATATAATTTTTAAA  480
GACAACTCAAGCCACAAATTCCATTAAAATGTAATGAATAAAGTAATCGTTTTTTTTTAT  540
TTGAATATTTTTTCTATTTTCTCATAATTATGTGTGCGACTGAGTGAATTTCATAGATGA  600
CCAGGGAAATCCAGTTCATAATCCCAAAATGTCAATGTTTCCATATATTCTTGTATAATG  660
TAAAATAATTAATATTTAAGAGCATTTAATAAATGTCAAAAAAAATTCATTCCCTTAATC  720
GATTGTAGATTTGAAAAAAAAAAAGGCACAATGTCCATAATCTATGGTCACTGGTGTATTT  780
TAAATGATTAAAATGAAATTATTCATTTTCACGTCACTAACGAAATAGTAGATCATATTT  840
TGTTTTGTTTAATTTTAATAATGTAATTTATTTGAATGAAATACAGTATATTTGTCAAAT  900
TAAAAAAAAAAAAAAAAA                                             919
```

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the *B. mori* RAMP4 gene. The predicted amino acid is represented by the one-letter code designation below the nucleotide sequence. The initiate and stop codes are framed and the putative TATA boxes and PolyA signal are underlined.

Genomic analysis

As the genomes of *B. mori* was released in the Genbank, we blasted the cDNA which we got to the contigs of *B. mori* genomes in Genbank to establish the DNA sequence of this gene, and the contig 1412 (Access Number in GenBank: BAAB01018328) which was homologous to the cDNA we got were identified. Using SlM4, we got three exons and two introns in the relevant DNA sequence (Fig. 2), and the 1st and 2ed exons are relative short (70bp and 114bp, respectively), while the 3rd exon is longer(816bp). Splice signals (exon / GU-intron-AG / exon) were identified by NNSSP and showed in Fig. 2. Meanwhile, other splice signals that won't relevant to our cDNA were predicted in the DNA sequence too. As the cDNA sequence has only high similarity to the contig 1412, it is also can be established that the RAMP4 gene of *B. mori* is a single copy gene through Genomes.

Promoter and poly-A signals prediction

A putative promoter (in -141 to -91 of DNA sequence, the ATG was defined as position 1) was identified by the Neural Network Promoter Prediction (NNPP). Using HCtata and another TATA-box prediction tool, we found the TATA-boxes (ATTATTAAAC and ACTTTATAAC) of this gene (Fig. 1). PolyA signal (ATTAAA) of this gene was found at position 787 by PolyA Scan (Fig. 1). This signal was also identified when Hcpolya was used.

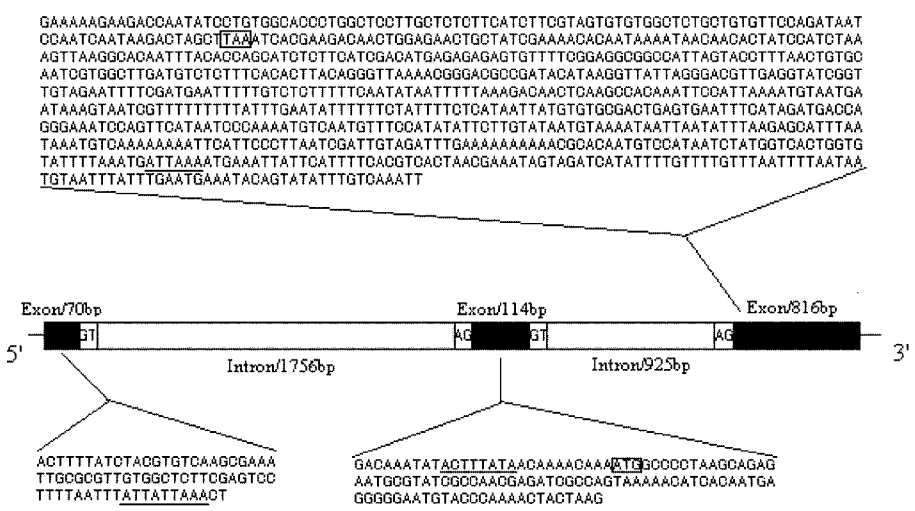

Fig. 2. DNA sequence frame of the RAMP4 gene. It was based on contig 1412 (GenBank access in number: BAAB01018328). The splicing signals (exon / GU-intron-AG / exon) have been indicated.

Amino acid sequence analysis

The peptide encoded by the 195 bp ORF was a putative RAMP4, which has 64 amino acid residues. By SOSUI, this amino acid sequence was predicted as a membrane protein which has a putative transmembrane-spanning domain at the COOH (Fig. 3), and the transmembrane helix has 23 residues from N terminal 39 to C terminal 61. No signal peptide was found by SignalP 3.0 Server used the neural networks method, while a signal anchor was got used the hidden Markov models and the max cleavage site probability was 0.001 between position 53 and 54

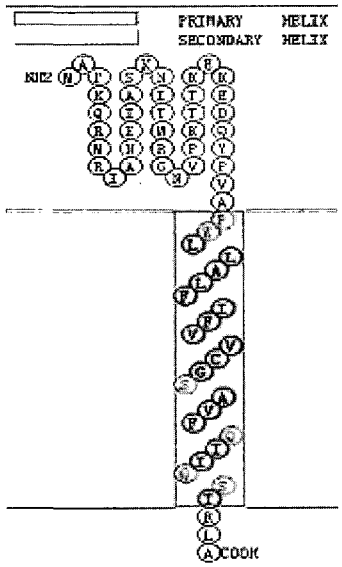

Fig. 3. Ransmembrane domain of *B. mori* pupative RAMP4, and the transmembrane helix was in shadow. Predicted by SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html).

Sequences of the amino acid were Aligned with those of 14 other organisms using DNAstar ClUSTAL W program. Based on sequence homology database searches, the sequence of RAMP4 of *B. mori* shares 81.3%, 79.7%, 70.3%, 68.8%, 68.8%, 68.8%, 67.2%, 67.2%, 64.1%, 60.9%, 57.8%, 42.2%, 40.6%, 40.6% similarity with the RAMP4 of *Anopheles gambiae, Drosophila melanogaster, Homo sapiens, Mus musculus, Rattus norvegicus, Xenopus laevis, Pongo pygmaeus, Danio rerio, Tetraodon nigroviridis, Caenorhabditis elegans, Caenorhabditis briggsae, Phaseolus vulgaris, Arabidopsis thaliana, Plasmodium yoelii,* respectively (Fig. 4). Among the 64 amino acid residues, 12 and 13 amino acid residues are different from *A. gambiae* and *D. melanogaster*, respectively (Fig. 5). However the similarity with the RAMP4 of *P. yoelii* which belongs to protozoan was only 40.6% (Fig. 4). While aligned *B. mori* RAMP4 to the other species (belong to insecta, vertebrata, viridiplantae and protozoan, respectively), it could be found that the similarity reduced gradually, accordingly.

This is the first case of ribosome-associated membrane protein 4 (RAMP4) gene reported in *B. mori*. As the RAMP4 gene is overexpressed in silkworm strain 306 exposured to BmNPV and RAMP4 is involved in protein translocation across the membrane of the endoplasmic reticulum (Wang and Dobberstein, 1999), it is indicated that RAMP4 of *B. mori* may be associated with the infection process of BmNPV. While BmNPV can be biologic stress, *B. mori* RAMP4 is also may be involved in the

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1		81.3	40.6	57.8	60.9	79.7	67.2	70.3	68.8	42.2	40.6	67.2	68.8	64.1	68.8	1
2	21.6		40.6	56.3	56.3	82.8	75.0	76.6	75.0	42.2	43.8	76.6	78.1	68.8	78.1	2
3	93.3	98.3		33.8	35.4	37.5	40.9	43.1	41.5	88.2	49.3	39.4	37.9	37.9	37.9	3
4	61.2	64.6	117.5		95.4	56.3	55.4	60.0	58.5	33.8	33.8	52.3	52.3	50.8	53.8	4
5	54.7	64.6	117.5	4.8		54.7	55.4	60.0	58.5	35.4	33.8	52.3	52.3	50.8	53.8	5
6	23.7	19.6	109.1	64.6	68.2		67.2	67.2	65.6	39.1	39.1	67.2	67.2	65.6	67.2	6
7	43.0	30.4	100.5	66.5	66.5	43.0		89.2	87.7	40.9	34.8	86.4	87.9	72.7	89.4	7
8	37.8	28.2	90.7	56.6	56.6	43.0	9.9		98.5	44.6	36.9	87.7	89.2	78.5	89.2	8
9	40.4	30.4	95.5	59.8	59.8	45.8	11.7	1.6		43.1	35.4	86.2	87.7	76.9	87.7	9
10	88.6	93.3	12.8	117.5	117.5	103.6	95.5	86.2	90.7		46.3	40.9	39.4	39.4	39.4	10
11	109.1	98.3	86.0	138.3	138.3	115.1	123.9	123.9	130.8	90.4		40.9	39.4	33.3	37.9	11
12	43.0	30.4	100.5	77.8	77.8	45.8	15.1	11.7	13.5	95.5	117.5		98.5	74.2	89.4	12
13	40.4	28.2	105.8	73.9	73.9	43.0	13.3	9.9	11.7	100.5	123.9	1.5		75.8	90.9	13
14	48.6	40.4	105.8	81.9	81.9	45.8	33.9	25.4	27.6	100.5	155.2	31.6	29.3		75.8	14
15	40.4	25.9	105.8	70.1	70.1	43.0	11.5	11.7	13.5	100.5	117.5	11.5	9.7	29.3		15
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	

Species	GenBank No.
B. mori	This Study
A. gambiae	EAA43169
A. thaliana	NP_564279
C. briggsae	CAE69736
C. elegans	NP_510604
D. melanogaster	NP_728830
D. rerio	AAH49018
H. sapiens	CAI12995
M. musculus	AAR01199
P. vulgaris	AAQ09002
P. yoelii	EAA16367
P. pygmaeus	CAH89666
R. norvegicus	AAH61854
T. nigroviridis	CAF89799
X. laevis	AAH78563

Fig. 4. Pairwise identitied and similarlities of amino acid sequence of 15 species (percent similarity in upper triangle). The abbreviation of species name and GenBank accession number for RAMP4 sequence were given.

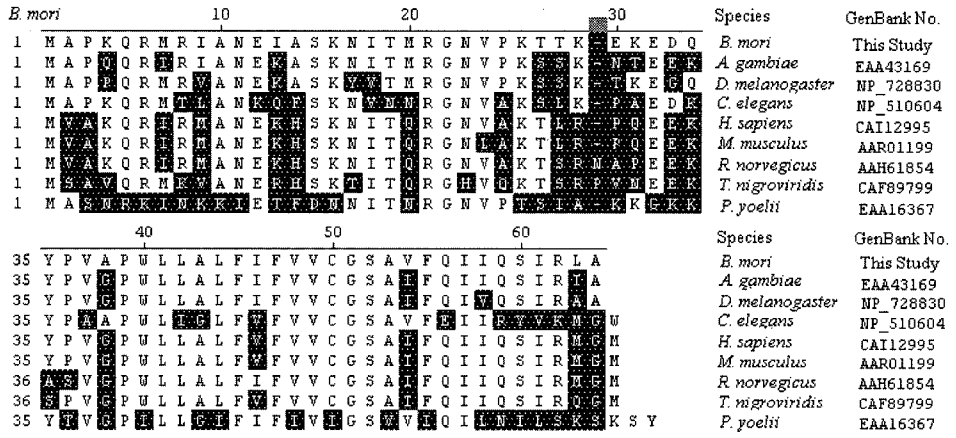

Fig. 5. Comparison of deduced amino acid sequences of RAMP4 in 9 species by DNAstar software. The template residue was RAMP4 sequence of *B. mori*. Residues differing from *B. mori* were in black shading. The abbreviation of species name and GenBank accession number for RAMP4 sequence were given.

resistance of *B. mori* to BmNPV, since the excessive RAMP4 may induce unnatural response of cell, even cell apoptosis. To determine the detailed function of RAMP4 in the context of biologic stress, further research work such as expression, functional assay, catalyzing activity and mutagenesis of this gene are under our consideration.

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