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Identification of genes expressed preferentially in the honeybee mushroom bodies by combination of differential display and cDNA microarray[1]

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Abstract To clarify the molecular basis underlying the neural function of the honeybee mushroom bodies (MBs), we identified three genes preferentially expressed in MB using cDNA microarrays containing 480 differential display-positive candidate cDNAs expressed locally or differentially, dependent on caste/aggressive behavior in the honeybee brain. One of the cDNAs encodes a putative type I inositol 1,4,5-trisphosphate (IP_3) 5-phosphatase and was expressed preferentially in one of two types of intrinsic MB neurons, the large-type Kenyon cells, suggesting that IP_3-mediated Ca^{2+} signaling is enhanced in these neurons.

Key words: Honeybee; Mushroom body; cDNA microarray; Differential display; Inositol 1,4,5-trisphosphate 5-phosphatase; Brain

1. Introduction

Mushroom bodies (MBs) are involved in higher-order sensory processing for different sensory modalities and are important structures for memory formation in the insect brain [1–4]. The MBs of the honeybee are more prominent compared with those of most other insects. In the honeybee, each MB has two calyces composed of 170 000 Kenyon cells, the intrinsic MB neurons [5,6]. In contrast, the fruit fly has one calyx and there are approximately 2500 Kenyon cells per hemisphere [7]. The honeybee MBs have a high degree of structural plasticity and the volume of the neuropil varies according to sex and division of labor of the workers [5,8]. These observations suggest that MB function is closely associated with the social behaviors of the honeybee [9].

The honeybee MBs are composed of two morphologically distinct large- and small-type Kenyon cells [6]. The large-type Kenyon cells are located on the inside edges of each calyx and receive projections from olfactory and optic neurons at the lip and collar zones of the calyces, respectively [6]. Thus, the large-type Kenyon cells might be involved in higher-order processing of olfactory or optic information.

We previously searched for gene(s) expressed preferentially in the MBs of the worker honeybee *Apis mellifera* L. brain using the differential display method [10] and attempted to identify gene(s) involved in the intrinsic function of the MBs [11,12]. By screening approximately 1000 bands with 20 primer combinations, we found that the inositol 1,4,5-trisphosphate (IP_3) receptor gene [11] and the Mblk-1 (MB large-type Kenyon cell-specific protein-1) gene [12], which encodes a putative transcription factor similar to *Drosophila* E93 [13], are expressed preferentially in the MBs. Expression of genes for two other proteins involved in the Ca^{2+} signaling pathway, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C, is also concentrated in the MBs of the honeybee brain [14]. In the MBs, the genes for the IP_3 receptor and CaMKII are expressed preferentially in the large-type Kenyon cells [11,14]. The gene for cAMP-dependent protein kinase A is also expressed preferentially in the large-type Kenyon cells [15]. These proteins are components of the Ca^{2+} or cAMP signaling pathways, both of which are involved in synaptic plasticity and learning [16,17]. Thus, we proposed that expression of some of the genes involved in neural plasticity is enhanced in the honeybee MBs, especially in the large-type Kenyon cells [12,14].

In the present study, to further identify gene(s) involved in honeybee MB function, we searched for gene(s) expressed preferentially in the MBs using a combination of the differential display method and cDNA microarray analysis.

2. Materials and methods

2.1. RNA extraction and differential display

Three types of genes were examined using the differential display method. Type 1 genes are those whose expression is enhanced in either of the three brain regions (MBs, optic lobes (OLs), and antennal lobes (ALs)) of the worker bees. Type 2 genes include those whose expression differs in either of the above three brain regions between the guard bees that patrol the hive entrance (aggressive bees) [9] and other non-aggressive worker bees. Type 3 genes are those whose expression differs in the MBs depending on the caste of the honeybee. For type 1 and 2 genes, MBs, OLs, and ALs were dissected from the aggressive and non-aggressive workers in the hive. For type 3 genes, MBs were dissected from the heads of the queens and worker bees.

Total RNA (2 μg) extracted from these tissues was treated with 2 U

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of RNase-free DNase I and then reverse-transcribed with 200 U of SuperScriptII RT (Invitrogen). The differential display method was performed using a Fluorescence Differential Display kit (TaKaRa) with 216 primer combinations.

2.2. Preparation of cDNA microarrays

A total of 80 candidate bands identified using the differential display method were randomly selected and excised from the gel. The cDNAs were re-amplified, subcloned into a pGEM-T vector (Promega), and transfected into *Escherichia coli* DH5α (TaKaRa). Six transformants per band were picked and suspended in 100 μl of LB broth. 5 μl of each was used as a template for polymerase chain reaction (PCR) and the remainder was mixed with 100 μl of 75% glycerol and stored at −80°C. cDNA inserts were amplified by PCR using the M13 forward (5′-CGCCAGGGTTTTCCCAGTCACG-3′) and reverse (5′-TCACACAGGAAACAGCTATGAG-3′) primers, which had amino-modified 5′-ends. The PCR conditions were: (94°C for 30 s, 49°C for 30 s, 72°C for 1.5 min)×35 cycles. A part of the resulting PCR products was subjected to 1.2% agarose gel electrophoresis to confirm re-amplification. The remaining samples were precipitated with isopropanol and the precipitates were washed with 75% isopropanol. The samples were then dissolved in 5 μl TE (10 mM Tris–HCl, pH 7.4, and 1 mM EDTA), mixed with 5 μl of solution X (DNA Chip Research Institute) and transferred to a 96-well plate. The cDNAs were printed onto polylysine-coated slides (DNA Chip Research Institute) using SPBIO-2000 (Hitachi Software Engineering Co., Ltd.).

2.3. Hybridization and detection in cDNA microarray analysis

Total RNA was extracted from the MBs and OLs of approximately 1000 workers and poly(A)$^+$ RNA was isolated using Oligotex-dT30 Super (TaKaRa). Poly(A)$^+$ RNA (2 μg) was reverse-transcribed with random primers using an RNA Fluorescence Labeling kit (TaKaRa). The probes, labeled with Cy3 or Cy5, were mixed and then purified using a Microcon-30 (Millipore). Following hybridization and washing, the expression level of each clone was measured using Scan Array 4000 (GSI Lumonics) and the Cy3/Cy5 fluorescence ratio was compared for each target cDNA. The difference between intensities of Cy3- and Cy5-derived signals was normalized to that of the honeybee β-actin signals [18]. Genes whose expression was reproducibly detected at least more than two-fold in the MBs than in the OLs in duplicate hybridization reactions using the same RNA preparation were assigned as positive clones.

2.4. cDNA cloning and in situ hybridization analysis of IP_3 5-phosphatase

To obtain a full-length cDNA for IP_3 5-phosphatase, 5′-rapid amplification of cDNA (RACE) was performed with 2 μg of poly(A)$^+$ RNA extracted from the worker bee MBs using a Marathon cDNA amplification kit (Clontech). Phylogenetic analysis was conducted using the neighbor-joining method implemented in the CLUSTAL W program [19]. Confidence levels were estimated using the bootstrap resampling procedure (1000 resamplings). In situ hybridization was performed as described previously [11,12,14].

3. Results

3.1. Screening of genes expressed preferentially in the honeybee MBs using cDNA microarray

Thus far, we identified approximately 400 candidate bands for type 1 genes using the differential display method. We also identified approximately 450 candidate bands for type 2 genes and 350 candidate bends for type 3 genes. Type 1 genes are those whose expression is enhanced in either of the three brain regions (MBs, OLs, and ALs) of the worker bees. Type 2 genes include those whose expression differs in either of the above three brain regions between the aggressive and non-aggressive worker bees. Type 3 genes are those whose expression differs in the MBs depending on the caste of the honeybee.

We intended to use cDNA microarray analysis to confirm the MB-preferential expression of the type 1 candidate clones as well as to search for genes expressed preferentially in the MBs among type 2 or 3 candidate clones. A total of 80 candidates from the more than 1100 candidate bands, 25 for type 1, 45 for type 2, and 10 for type 3 genes, were randomly selected (Table 1). Cloning the DNA from a band identified using the differential display method frequently resulted in isolation of a mixture of cDNA subclones because it was difficult to avoid contamination of neighboring bands during band excision. Thus, we isolated six independent subclones derived from each band (total 480 clones derived from 80 bands) and printed them on the microarrays. We also printed cDNA fragments for honeybee β-actin [14,18] as a control, which was expressed almost equally in all samples tested, and those for the IP_3 receptor [11] and Mblk-1 [12] as controls for genes expressed preferentially in the MBs, in triplicate.

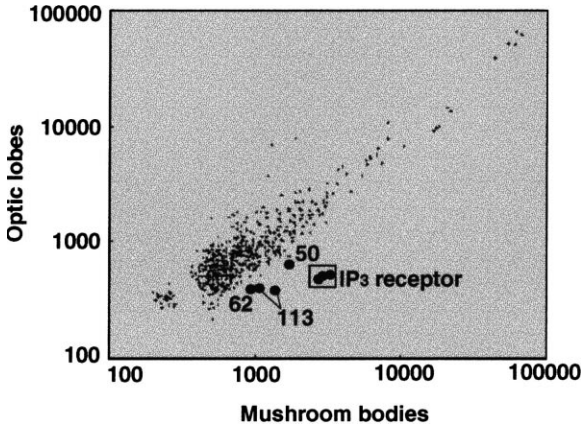

Fig. 1. Summary of the microarray analysis. A total of 480 clones were spotted, representing six clones from each of 80 candidate bands identified using the differential display method. Horizontal and vertical lines indicate expression levels of each clone in logarithmic scales. Each dot represents MB- versus OL-preferential expression of a single clone. Closed circles indicate positive clones that are expressed preferentially in the MBs. Among them, two no. 113 clones are indicated by arrows. The IP_3 receptor cDNA clones, which were spotted in triplicate and used as positive controls, are boxed.

The results are summarized in a scatter plot where the expression of each clone in the MBs versus that in the OLs is represented as dots (Fig. 1). Most of the dots are positioned along a diagonal line, indicating that they are expressed almost equally in both tissues. The expression of three clones (no. 50, 62, and 113) and IP_3 receptor cDNA, however, was augmented by more than two-fold in duplicate hybridization reactions (Table 2). The expression level of the Mblk-1 gene

Table 1
Summary of candidate bands identified using the differential display method

Expression	Number of candidate bands
Type 1. Local expression in the worker bee brain	
(1) MBs	18
(2) OLs	4
(3) ALs	3
Type 2. Aggressive behavior-selective expression	
(1) Aggressive bees	32
(2) Non-aggressive bees	13
Type 3. Caste-selective expression	
(1) Queen	7
(2) Worker	3

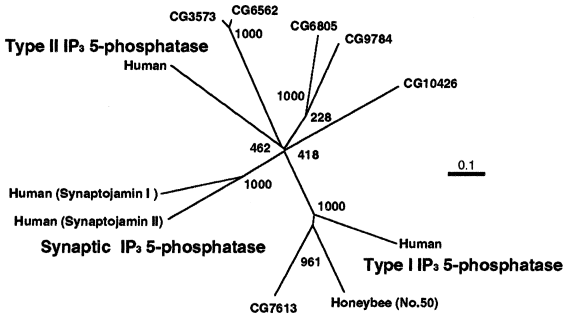

Fig. 2. Phylogenetic tree displaying the relation between honeybee, fly, and human IP_3 5-phosphatases. CG7613, CG10426, CG9784, CG6805, CG6562, and CG3573 correspond to six predicted IP_3 5-phosphatases registered in the fly genome database. GenBank/EMBL accession numbers are: human type I IP_3 5-phosphatase, X77567; human type II IP_3 5-phosphatase, M74161; synaptojanin I, AF009040; synaptojanin II, AF039945.

did not reach the threshold for detection using the array method (data not shown). Of the positive clones, clones no. 50 and 113 were derived from candidate bands preferentially detected in the MBs (type 1 genes), whereas no. 62 was derived from a candidate band preferentially detected in the ALs of the aggressive bees (type 2 genes).

3.2. *cDNA cloning and in situ hybridization analysis of the no. 50 (IP_3 5-phosphatase) gene*

To identify proteins encoded by clones no. 50, 62, and 113, the nucleotide sequences of these cDNA fragments were determined (Table 2). Some stop codons appeared in both strands of clones no. 62 and 113 (222 and 270 bp, respectively) and there was no obvious open reading frame (ORF) for these clones (Table 2). Clone no. 50 (252 bp) contained an ORF and thus 5′-RACE was performed to isolate its full-length cDNA. As a result, this cDNA was determined to encode a protein composed of 388 amino acid residues. A database search (FASTA) revealed that the highest sequence similarity (51% identical amino acids) was between this protein and human type I IP_3 5-phosphatase [20,21]. Furthermore, a phylogenetic analysis including clone no. 50 protein, six predicted IP_3 5-phosphatases found in the *Drosophila* genome database [22], and four mammalian IP_3 5-phosphatases [23–25] revealed a clear clustering of this protein, *Drosophila* CG7613 protein, and human type I IP_3 5-phosphatase (Fig. 2). This finding indicates that clone no. 50 protein is a type I IP_3 5-phosphatase and CG7613 protein is the *Drosophila* orthologue of this protein.

To confirm that the IP_3 5-phosphatase gene is expressed preferentially in the MBs, in situ hybridization analysis was performed using frozen sections of the worker brain with a digoxigenin-labeled antisense RNA probe. Strong expression was observed in cell bodies of the large-type Kenyon cells, which are located at the inside edges of each MB calyx (Fig. 3A,B,D). Furthermore, there was weak expression in cell bodies of the small-type Kenyon cells, which are located on the inner core of the inside of each calyx (Fig. 3B). Very weak or basal levels of expression were observed in the cortex of all the other brain regions, such as the OL or AL (Fig. 3A). There was no significant signal detected in the sections hybridized with sense RNA (Fig. 3C), indicating that the signal was due to IP_3 5-phosphatase mRNA.

4. Discussion

The present paper demonstrated that the gene for IP_3 5-phosphatase is expressed preferentially in the large-type Kenyon cells of the honeybee brain. This is the first demonstration of the expression pattern of the IP_3 5-phosphatase gene in the insect brain. In mammalian brain, type I IP_3 5-phosphatase is the major isoenzyme hydrolyzing the Ca^{2+}-mobilizing second messenger IP_3 [20]. In cerebellar Purkinje neurons, the IP_3 receptor and type I IP_3 5-phosphatase genes are co-expressed [21,26] and IP_3-mediated Ca^{2+} signaling in dendritic spines is involved in the induction of long-term synaptic depression [27]. We previously demonstrated that the IP_3 receptor [11] and CaMKII [14] genes are expressed preferentially in the large-type Kenyon cells of the honeybee brain and proposed that Ca^{2+} signaling and, thus, synaptic plasticity of the large-type Kenyon cells, are enhanced in the honeybee brain. The present results further strengthen this hypothesis. The other two clones contained no significant ORFs, suggesting that these clones corresponded to 3′-untranslated regions of certain genes.

Both clones no. 50 (IP_3 5-phosphatase) and no. 113 were derived from candidate bands identified using the differential display method for type 1 genes with MB-preferential expression. Thus, two of 18 differential display-positive bands (approximately 11%) were confirmed to be expressed preferentially in the MBs using cDNA microarray analysis. Clone no. 62 seemed to be isolated incidentally, as it was derived from the type 2 genes, which are detected preferentially in the ALs of aggressive bees. Considering that the differential display method yields false positives as often as two to four times that of the positive clones [28–30], the combined use of the differential display method and cDNA microarray analysis is an excellent method for further large-scale screening of genes expressed preferentially in the MBs. The microarray technique has already been applied to screen clones isolated through a subtractive hybridization procedure [31]. As MB-preferential expression of the Mblk-1 gene was not detected in the present experiment, some genes with relatively low expression levels

Table 2
Summary of the genes expressed preferentially in the MBs

Clone no.	Homology	Relative expression levels[a] (-fold)	Accession number
No. 50	type I IP_3 5-phosphatase	2.55 ± 0.15	AB072429
No. 62	ND[b]	2.50 ± 0.20	AB072427
No. 113	ND	3.00 ± 0.31	AB072428
Positive control	IP_3 receptor	5.20 ± 0.30	AB006152

[a]The average relative expression level of each clone in the MBs compared to that in the OLs in duplicate hybridization experiments is indicated with standard error.
[b]ND, not detected.

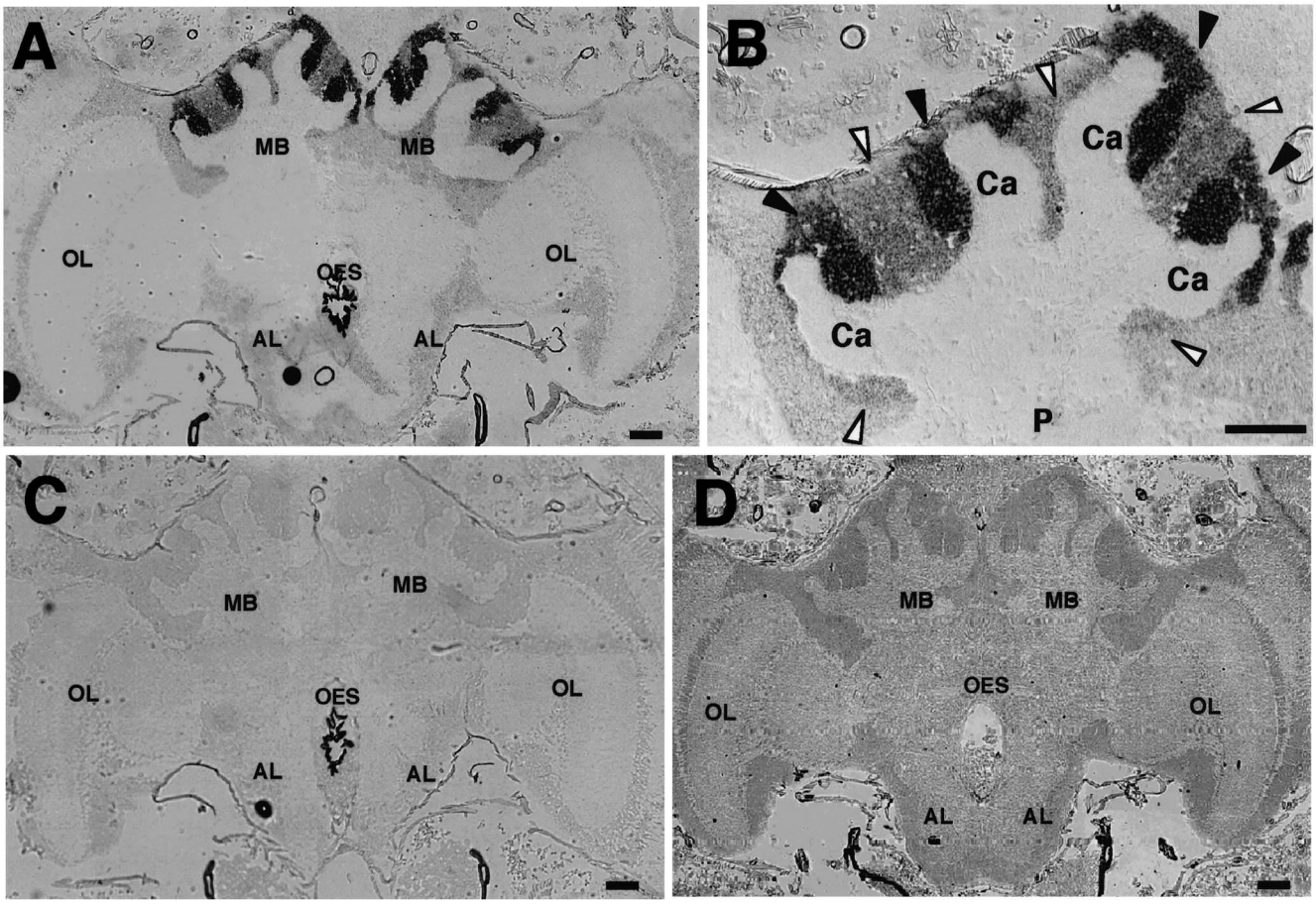

Fig. 3. Expression of the IP_3 5-phosphatase gene in the worker bee brain. A: Frontal section of a worker bee brain hybridized with digoxigenin-labeled antisense RNA for the IP_3 5-phosphatase cDNA. B: High-magnification micrograph of the left MB shown in (A). The large- and small-type Kenyon cells are indicated by black and white arrowheads, respectively. C: Control experiment with digoxigenin-labeled sense RNA. D: Whole brain stained with eosin to show the localization of cell bodies. Ca, calyx, which is formed by dendrites of Kenyon cells; P, peduncle, which is formed by axons of Kenyon cells; OES, oesophagus. Bars indicate 0.1 mm.

could have been overlooked. Hundreds of candidate clones can be screened in a single experiment using this method, overcoming the disadvantages in its relatively low sensitivity, at least in the first screening of relatively abundant candidate genes. The method is applicable not only for large-scale screening of behavior-associated genes but also for analysis of the relation between region-specific genes and behavior-associated genes in the honeybee brain.

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