Rab proteins in the brain and corpus allatum of *Bombyx mori*

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Abstract In eukaryotic cells, Rab guanosine triphosphate-ases serve as key regulators of membrane-trafficking events, such as exocytosis and endocytosis. Rab3, Rab6, and Rab27 control the regulatory secretory pathway of neuropeptides and neurotransmitters. The cDNAs of Rab3, Rab6, and Rab27 from *B. mori* were inserted into a plasmid, transformed into *Escherichia coli*, and then subsequently purified. We then produced antibodies against Rab3, Rab6, and Rab27 of *Bombyx mori* in rabbits and rats for use in western immunoblotting and immunohistochemistry. Western immunoblotting of brain tissue revealed a single band at approximately 26 kDa. Immunohistochemistry results revealed that Rab3, Rab6, and Rab27 expression was restricted to neurons in the pars intercerebralis and dorsolateral protocerebrum of the brain. Rab3 and Rab6 co-localized with bombyxin, an insect neuropeptide. However, there was no Rab that co-localized with prothoracotropic hormone. The corpus allatum secretes neuropeptides synthesized in the brain into the hemolymph. Results showed that Rab3 and Rab6 co-localized with bombyxin in the corpus allatum. These findings suggest that Rab3 and Rab6 are involved in neurosecretion in *B. mori*. This study is the first to report a possible relationship between Rab and neurosecretion in the insect corpus allatum.

Keywords *Bombyx mori* · Rab · Brain · Insect · Corpus allatum · Bombyxin

Introduction

Rab proteins are small (21–25 kDa) monomeric guanosine triphosphate (GTP)-ases/GTP-binding proteins that play significant roles in the regulation of various trafficking pathways (Hutagalung and Novick 2011; Bhuin and Roy 2014; Barr 2013). For example, Rab1 regulates membrane trafficking within early Golgi compartments and in the ER–Golgi transition (Plutner et al. 1990, 1991; Sannerud et al. 2006). Rab7 is associated with both the endosome and lysosome and has been shown to facilitate endosomal maturation and transport from the late endosome to the lysosome (Bucci et al. 2000; Wang et al. 2012). Rab14 has been found on endosomes and Golgi membranes in several cell types (Junutula et al. 2004; Proikas-Cezanne et al. 2006), localizes in the endolysosomal pathway, and regulates phagosome–lysosome fusion (Harris and Cardelli 2002).

In *Drosophila melanogaster* and *Helicoverpa armigera*, Rab proteins regulate important cellular processes, such as synaptic functions, midgut remodeling, oogenesis, autophagy, and the immunological system (Chan et al. 2011; Coutelis and Ephrussi 2007; Tong et al. 2011; Hou et al. 2011; Wang et al. 2012; Li et al. 2015). However, very little is known about the relationship between neuropeptide secretion and Rab in insects.

In the brain of *Bombyx mori*, the silkworm, immunohistochemistry results have shown that Rab1, Rab7, and Rab14 co-localizes with bombyxin, an insect neuropeptide.
(Uno et al. 2013). Rab7 and Rab1 have also been shown to co-localize with eclosion hormone, and Rab1 co-localizes with prothoracicotrophic hormone (PTTH). Rab1, Rab7, and Rab14 are involved in neuropeptide transport in neurons in the B. mori brain and thought not to relate to exocytosis. The role that Rabs play in exocytosis from neurons via neurohemal organs into the hemolymph remains poorly understood.

Rab3, Rab6, and Rab27 function in the process of regulatory exocytosis (Bhun and Roy 2014). Rab6 plays a key role in membrane trafficking around the Golgi complex. Rab6 cooperates with various effector proteins, including motor proteins to regulate fission of transport vesicles from the Golgi and translocation of Golgi-derived vesicles along microtubules (Echard et al. 1998; Grigoriev et al. 2007, 2011). In Caenorhabditis elegans, Rab6 is required for timely exocytosis of cortical granules during oocyte-to-embryo transition (Kimura and Kimura 2012). Rab6 also regulates cell polarity in Drosophila oocytes (Januschke et al. 2007).

Rab3 is the most abundant Rab protein in the brain; it is localized in synaptic vesicles and participates in synaptic vesicle fusion and neurotransmitter release (Ng and Tang 2008; Shin 2014). In Bombbyx mori, RNAi knockdown of Rab3 leads to lethality of larvae and pupae (Singh et al. 2015).

Rab27b is expressed in synaptic vesicles isolated from the rat brain (Takamori et al. 2006) and is restricted to the central nervous system and enriched on synaptic vesicles (Pavlos and Jahn 2011; Zhao et al. 2002).

In C. elegans and D. melanogaster, Rab3 and Rab27 proteins have been linked to trafficking of synaptic vesicles (Barclay et al. 2012; Graf et al. 2009; Chan et al. 2011). However, the relationship between these secretory Rabs (Rab3, Rab6, and Rab27) and exocytosis, which leads to the release of neuropeptides into the hemolymph, remains to be determined.

Neuropeptides are small signaling molecules that play important roles in the development, physiology, and behavior of both vertebrates and invertebrates (Nassal and Homburg 2006; Nassel and Winther 2010; Hoyer and Bartfai 2012). Specifically, insect neuropeptides regulate or cause metamorphosis, molting, feeding, development, and ecysis.

In Bombyx mori, at least 37 genes are predicted to encode biologically active neuropeptides (Roller et al. 2008). These neuropeptides are synthesized in specific neurosecretory cells of the brain, transported along axons, and secreted from the corpus cardiacum or corpus allatum into the hemolymph.

The corpus allatum (CA) is an organ specific to insects that works together with associated nerves, and secretes juvenile hormones and neuropeptides into the hemolymph (Tobe and Pratt 1974).

The neuropeptide hormone, bombyxin, is an insulin-like peptide found in the order Lepidoptera (butterflies and moths). Bombyxin was first identified in the silkworm, Bombyx mori (Nagasawa et al. 1986), and later in the tobacco hornworm, Manduca sexta (Nijhout and Grunert 2002; Van de Velde et al. 2007). Bombyxin stimulates cell division and is a growth factor for wing imaginal disks in Precis coenia and M. sexta (Nijhout and Grunert 2002; Nijhout et al. 2007).

In all insects, PTTH acts on the prothoracic glands, which initiate ecdysone synthesis (Nagata et al. 2005). Ecdysones is converted to 20-hydroxyecdysone, which acts on numerous target tissues inducing gene expression related to the molting process (Thummel 2002). Bombyxin and PTTH are released from the CA into the hemolymph (Mizoguchi and Okamoto 2013).

However, the relationship between Rab and the transport of these neuropeptides from the insect brain via corpus allatum to hemolymph has not yet been studied.

Therefore, the present study aimed to investigate the relationship between 3 Rabs (Rab3, Rab6, and Rab27) and bombyxin, or PTTH, secretion in the brains and corpus allatum of B. mori. Antibodies to B. mori-derived Rabs were used to identify Rab-expressing cells, locate their regional distribution, and ultimately evaluate co-localization of Rabs with bombyxin and PTTH. This study pinpointed clarifying the anatomical relationships between Rabs and bombyxin secretion.

Materials and methods

Materials

The mRNA purification kit, pGEX6P2, and glutathione S-Sepharose were from GE Healthcare UK Ltd. (Little Chalfont, Bucks, UK). Donkey anti-mouse and antirat IgG (H+L)-CF™555 and donkey anti-rabbit IgG (H+L)-CF™488A were from Biotium Inc. (Hayward, CA, USA). The plasmid pCR2.2 was from Invitrogen (Tokyo, Japan). The peroxidase-conjugated goat anti-rat and anti-rabbit IgG were from Wako Pure Chemicals (Tokyo, Japan). The Blocking One solution, peroxidase and 3,3′-diaminobenzidine, were from Invitrogen (Tokyo, Japan). The Blocking One solution, peroxidase and 3,3′-diaminobenzidine, were from Invitrogen (Tokyo, Japan). The Aqua-Poly/Mount medium was from Polysciences Inc (PA, USA). The DX50 microscope was from Olympus (Tokyo, Japan). All chemicals used were of the purest grade commercially available.

Insect culture

We raised hybrids (Kinshu × Showa or Shunrei × Shogetsu) of B. mori; larvae were reared on an artificial diet (Silkmate 2 M, Nosan Co, Yokohama, Japan) at 25 °C and
subjected to a light–dark cycle of 16-h light/8-h dark with a relative humidity of 70%. Day 5 fourth-instar larvae were used in all experiments. Silkworms were kindly provided by Dr. Yoko Takemura, Institute of sericulture, Ibaraki, Japan.

**Purification of B. mori Rab6, Rab3, and Rab27**

The cDNA fragments containing the coding sequences of *B. mori* Rab3, *B. mori* Rab6, and *B. mori* Rab27 were generated by RT-PCR and then sub-cloned into pCR2.2. The plasmid pCR2.2 was digested with *BamHI* in combination with *EcoRI*. The digested fragments containing Rab3, Rab6, and Rab27 of *B. mori* were then inserted between the *BamHI* and *EcoRI* sites of pColdII or pGEX6P2. The plasmids pGEX6P2 and pColdII express the target protein as GST fusion protein and His-tagged protein, respectively. The identity of clones containing Rab3, Rab6, and Rab27 of *B. mori* was confirmed by sequence analysis using an ABI Prism 377 DNA Sequencer.

*Escherichia coli* cells (BL21 strain) were transformed with pColdII and then incubated in Luria–Bertani (LB) medium (3–4 h, 37 °C). The fusion protein was produced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG; 0.2 mM) to the culture followed by incubation (24 h, 20 °C). The cells expressing the recombinant fusion proteins were collected by centrifugation (5000 g, 10 min) and then stored (−80 °C). Frozen cells from 1 L of culture were suspended in 10 mL lysis buffer (50 mM Na2HPO4, 300 mM NaCl, and 10 mM imidazole, [pH 8.0]) and were then disrupted by sonication. The homogenate was then cleared by centrifugation (12,000 g, 40 min). The supernatant containing the fusion protein was applied to a cartridge pre-filled with 1 mL His-tagged Rab protein (1 or 3 mg) and Freund’s complete adjuvant. The rabbit or rat received three booster injections at 2-week intervals. The serum was isolated and tested for the presence of anti-*B. mori* Rab antibodies by immunoblotting. Antiserum of *B. mori* Rab6 was obtained from rat and rabbit. Antiserum against *B. mori* Rab1, Rab7, Bombyxin, and PTTH was obtained, as previously described (Uno et al. 2007).

**Western immunoblotting**

*B. mori* brains were homogenized in 50 mM HEPES-HCl (pH 7.5) containing 20 % glycerol, 1 mM EDTA, and 1 mM dithiothreitol. Protein samples (1 μg) from purified recombinant Rab protein or brain extracts were separated by SDS-PAGE and were subsequently transferred to a PVDF membrane. The membranes were blocked in Blocking One solution (60 min, room temperature [RT]) and then incubated (60 min, RT) with primary antibodies: anti-Rab3 rabbit serum (1:2000), anti-Rab6 rat or rabbit serum (1:2000), or anti-Rab27 rat serum (1:1000) in Tris-buffered saline (TBS; 50 mM Tris HCl, 50 mM NaCl [pH 8.0]) containing Blocking One solution. The membranes were then washed (3 ×) with TBS [including 0.05 % Tween-20 (v/v)], followed by incubation (60 min, RT) with secondary antibodies: peroxidase-conjugated goat anti-rat IgG or peroxidase-conjugated goat anti-rabbit IgG (both 1:2000). After the membrane was washed (3 × with TBS plus Tween 20), proteins were detected using a peroxidase DAB kit.

**SDS–polyacrylamide gel electrophoresis (PAGE)**

SDS-PAGE was performed according to the Laemmli method (Laemmli 1970) using a 4.5 % stacking gel/15 % separating gel (16 mA, 60 min). The proteins were visualized with CBB staining.

**Immunohistochemistry**

Insect heads and brain–corpora cardiaca–corpora allata complex were fixed (24 h at 4 °C) in Bouin fluid (saturated with 10 mL wash buffer (50 mM Tris–HCl, 300 mM NaCl [pH 8.0]). Bound proteins were eluted from the resin with 5 mL of 10 mM reduced glutathione.

Protein concentrations of samples were determined using the Lowry protein assay (Lowry et al. 1951) with albumin (Fraction V, Sigma-Aldrich, Japan, Tokyo, Japan) as the standard protein.
picric acid, formalin, and acetic acid at 15:5:1 by volume). Standard histochemical methods were used for tissue dehydration, embedding in paraplast, sectioning (9-μm-thick sections), deparaffinization, and rehydration. The insect head and brain–corpora cardiaca–corpora allata complex sections were washed (at RT) in distilled water and in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, [pH 7.4]) containing 0.3 % Triton-X100 (PBS-Tr), blocked (30 min, RT) with antibody dilution buffer (PBS-Tr containing 1.5 % goat serum), and incubated (overnight, 4 °C) in this dilution buffer with the primary antibodies: anti-Rab3 rabbit serum (1:500), anti-bombyxin mouse IgG (1:500), anti-Rab6 rat or rabbit serum (1:500), anti-Rab27 rat or rabbit serum (1:500), or anti-PTTH mouse IgG (1:250). After rinsing (3 × 10 min, RT) with PBS-Tr, the sections were incubated (1.5 h, RT) with 7.5 μg/mL secondary antibodies: donkey anti-rat or anti-mouse IgG (H + L)-CF™555 or goat anti-rabbit IgG (H + L)-CF™488A. After washing in PBS-Tr, the sections were mounted in Aqua-Poly/ Mount medium and examined using a BX50 microscope equipped with BX-FLA reflected light fluorescence and WIG and NIBA mirror/filter units. The number of individuals studied in each immunohistochemical experiment was three to five. Excitation wavelength and emission range in the WIG mirror/filter unit were 520–550 and >580 nm, respectively. Excitation wavelength and emission range in the NIBA mirror/filter unit were 470–490 and 515–550 nm, respectively.

In the control experiments, the primary antibodies were replaced with pre-immune rabbit or rat serum. As an additional control for binding specificity, the anti-Rab antibody was pre-incubated with an excess amount of antigen (100 mM) before immunohistological staining. In both cases, no significant staining was observed above background.

Results

Antibody production against B. mori Rab is specific

Antibodies produced against B. mori Rabs specifically recognized the protein band corresponding to the purified partial B. mori Rabs (Fig. 1, lanes 2, 6, and 10). Two control experiments (i.e., addition of pre-immune serum in place of primary antibody, and addition of antigen and primary antibody together) revealed no band (Fig. 1, lanes 3, 4, 7, 8, 11, and 12, respectively).

To examine whether these antibodies cross-react, B. mori Rab3, Rab6, and Rab27 proteins were generated as GST fusion proteins and purified. These proteins reacted specifically with their corresponding antibodies (Fig. 2). Anti-Rab3 reacted with Rab3 (Fig. 2, lane 1), but not with the other proteins (Fig. 2, lanes 2 and 3). Anti-Rab6 reacted with Rab6 (Fig. 2, lane 5), but not with the other proteins (Fig. 2, lanes 4 and 6). Anti-Rab27 reacted with Rab27 (Fig. 2, lane 9), but not with the other proteins (Fig. 2, lanes 7 and 8).

Rab3, Rab6, and Rab27 are expressed in the brain of B. mori

Western immunoblots for Rab3 and Rab6 revealed one band at approximately 25 kDa in the brain extract (Fig. 3, lanes 2 and 6), while no band was detected under control conditions (Fig. 3, lanes 3, 4, 7, and 8). Western immunoblots for Rab27 revealed one band and a faint band at
approximately 27 kDa in the brain sample (Fig. 3, lane 10), while no band was detected under control conditions (Fig. 3, lanes 11 and 12). Rab27 may be modified by phosphorylation or acylation.

**Rab3, Rab6, and Rab27 are expressed in specific neurons of the *B. mori* brain**

Anti-Rab3, anti-Rab6, and anti-Rab27 detected a restricted area in a set of neurons in the pars intercerebralis (PI) and dorsolateral protocerebrum (DL) (Fig. 4). Double-labeling experiments revealed Rab3 expression in a subset of Rab6-positive neurons (Fig. 4c, d, arrows). The stippled demarcations lines of brain are shown in Fig. 5b. Pars intercerebralis (PI) and dorsolateral protocerebrum (DL) in the brain and corpus cardiacum and corpus allatum on *Bombyx mori* are shown in Fig. 5c.

However, there was no Rab27 expression in Rab3- and Rab6-positive neurons (Fig. 4g, h, k, l). As a control for binding specificity, the anti-Rab6 antibody was pre-incubated with an excess amount of antigen before immunohistochemistry staining (Fig. 6a–c).
Rab3 and Rab6 expression in bombyxin-positive neurons of the brain

Bombyxin expression was detected in a restricted set of neurons in the PI area (Figs. 5c, 6e). This was consistent with previous findings in the silk moth (Mizoguchi et al. 1987). Double-labeling experiments showed Rab3 and Rab6 expression in bombyxin-positive neurons (Fig. 7c, d, g, h, arrows). However, there was no Rab27 expression in the bombyxin-positive neurons (Fig. 7k, l). Fluorescent bleed-through was evaluated by imaging single-stained samples through both filter/mirror units (WIG and NIBA). In both cases, there was no significant staining (Fig. 6d–i).
Rab3 and Rab6 exist in the periphery and bombyxin-positive cells of the corpus allatum

Rab3 and Rab6 were detected in the periphery of the corpus allatum. Double-labeling experiments revealed Rab3 expression in Rab6 neurons (Fig. 8o, p, arrows). Rab27 was not detected in the corpus allatum (Fig. 8s, t, w, x). Rab7 and Rab1 were also not detected (data not shown). And Rab3 and Rab6 existed in bombyxin-positive cells (Fig. 8c, d, g, h, arrows). However, Rab27 was not expressed in bombyxin-positive cells (Fig. 8k, l). These results suggest that Rab3 and Rab6 regulate secretion of bombyxin from the corpus allatum into the hemolymph.

Rab3 is not expressed in PTTH-positive cells of the corpus allatum

PTTH is released from the corpus allatum into the hemolymph (O’Brien et al. 1988). Double-labeling experiments showed that Rab3 was not expressed in PTTH-positive neurons of the corpus allatum (Fig. 9c).

Discussion

Rab proteins are master regulators of vesicular membrane trafficking in endocytosis and exocytosis pathways, primarily serving to recruit proteins and lipids required for vesicle formation, docking, and fusion. Each Rab protein recruits and drives action of one or more effectors for specific downstream functions (Schwartz et al. 2007; Stenmark 2009).

Neuropeptides play important roles in development, physiology, and behavior of invertebrates. Insect neuropeptides are synthesized in specific neurosecretory cells, transported along axons, and secreted from the corpus cardiacum or corpus allatum into the hemolymph. In this study, we aimed to clarify the relationship Rabs and neuropeptide secretion from the brain to the hemolymph in B. mori.

Our initial results show that Rab3, Rab6, and Rab27 proteins are expressed in the B. mori brain. Expression of Rab3, Rab6, and Rab27 is restricted to a subset of neurons in the pars intercerebralis (PI) and dorsolateral protocerebrum (DL). Furthermore, Rab3 and Rab6 co-localize with bombyxin in the PI. The PI and DL contain important neurosecretory centers in the insect brain (Hartenstein 2006). Thus, in the insect brain, co-localization of Rab3 and Rab6 and co-localization with bombyxin suggest the involvement of Rab3 and Rab6 in bombyxin secretion.

Bombyxin, a member of the insulin family of peptides, is synthesized in the insect brain (Nagasawa et al. 1986; Mizoguchi et al. 1987; Nagata et al. 1992; Iwami et al. 1996), axonally transported to the corpus allatum, and
Co-localization of BRab6, BRab3, BRab27, and bombyxin expression in the B. mori allatum. Rab3, Rab6, and Rab27 were visualized with CPTM488A (green fluorophore, a, e, i, m, q, u). Rab6, Rab27, and bombyxin were visualized with CPTM555 (red fluorophore, b, f, j, n, r, v). Rab3 was expressed in Rab6-positive cells (o, p; arrows). However, Rab27 was not expressed in Rab6- or Rab3-positive cells (s, t, w, x). Rab3 and Rab6 were detected in bombyxin-positive cells (c, d, g, h; arrows). However, Rab27 was not expressed in bombyxin-positive cells (k, l). Bar 100 μm, except d, h, i, p, t, x (bar 50 μm).

Our results show that Rab3 and Rab6 proteins existed in the corpus allatum of B. mori. Furthermore, Rab3 and Rab6 co-localize with bombyxin in the corpus allatum, respectively. Corpus allatum functions as an organ to secrete neuropeptides synthesized in the insect brain into the hemolymph (Tobe and Pratt 1974). Thus, co-localization of Rab3 and Rab6 with bombyxin in the insect corpus allatum suggests the involvement of these Rabs in bombyxin secretion. Rab3 is involved in the late steps of exocytosis (Graf et al. 2009). The basic and ancestral role of Rab3 was to promote neurotransmitter release, possibly by acting on recruitment or tethering of synaptic vesicles at the active zone. Rab6 regulates transport pathways into and out of the Golgi apparatus (Grigoriev et al. 2007). As a result, Rab6 may regulate transport from bombyxin-producing neurons in the brain via axons to the corpus allatum. Additionally, Rab3 may be involved in terminal secretion of bombyxin at the plasma membrane in the corpus allatum. Further studies are in progress to determine the functional role of Rab3 and Rab6 in the process of bombyxin secretion by examining the specific effector of Rab3 and Rab6.

Bombyxin secretion into the hemolymph is mediated by nutrient levels. (Tobler and Nijhout 2010; Masumura et al. 2000). Furthermore, an insulin-like peptide is released from the brain complex with a daily rhythm in both adult and larva Rhodnius prolixus (Hemiptera) (Vafopoulou and Steel 2012). Rab3 and Rab6 may regulate bombyxin secretion into the hemolymph according to levels of circulating nutrients, and expression might correlate with circadian rhythmicity of neuropeptides. Further studies are needed to determine whether Rab regulatory systems exist.

Rab27 expression is restricted to a subset of neurons in the PI and DL. However, Rab27 does not co-localize with bombyxin expression in the PI (Fig. 7), nor is it present in the corpus allatum (Fig. 8). Rab27 may regulate synthesis, transport, and secretion of some neuropeptides other than bombyxin, in the B. mori brain.

In mammals, Rab3A and Rab27 work together to coordinate synaptic vesicle (SV) docking and fusion. In particular, these two proteins act in a Rab cascade, functioning in successive steps of the SV release cycle. This hypothesis is supported by the observation that Rab3 and Rab27 share common interacting proteins (RIM, Munc-13) involved in docking and tethering of SVs (Pavlos and Jahn 2011). In B. mori, Rab3 does not co-localize with Rab27 (Fig. 4), suggesting that insect Rab3 and Rab27 do not work together to coordinate SV docking and fusion. Further studies are in progress to examine the localization of common interacting proteins (RIM and Munc-13) involved in docking and tethering of SVs.

PTTH is a critical neurohormone that regulates postembryonic development in insects by stimulating ecdysteroid secretion from the prothoracic glands (Agui et al. 1979). PTTH is produced by a pair of lateral neurosecretory cells in the protocerebrum of some insects (Zavodska et al. 2003). Our results indicate that Rab3 does not co-localize with PTTH. However, it remains to be determined whether other Rab proteins play a role in PTTH secretion.

Rab3 expression does not overlap with Rab6-positive cells in the corpus allatum. It is known that there are many neuropeptides in the corpus allatum (Vullings et al. 1999). Possibly, Rab3 and Rab6 may regulate transport and secretion of other neuropeptides in the corpus allatum.
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