Amino Acids (2011) 40:953–961
DOI 10.1007/s00726-010-0720-2

ORIGINAL ARTICLE

Rab32 and the remodeling of the imaginal midgut in *Helicoverpa armigera*

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Received: 9 April 2010 / Accepted: 4 August 2010 / Published online: 20 October 2010

Abstract Midgut remodeling is a complex physiological process in holometabolous insects. During midgut remodeling, the larval midgut is decomposed by apoptosis or autophagy during metamorphosis, and the degraded larval midgut is partially absorbed as nutrients by the imaginal midgut for its formation. The molecular mechanism involved in this process is not clear. Here, we found that a Rab protein, which we have named *HaRab32*, is related to the organogenesis of insect imaginal midgut. Results show that *HaRab32* is up-regulated in epidermis and midgut during metamorphosis. Its expression could be up-regulated by 20E. Immunohistochemistry shows Rab32 is distributed in the epithelium of the imaginal midgut during metamorphosis. Knockdown of *HaRab32* by RNA interference disturbs the formation of the imaginal midgut. These data imply *HaRab32* plays important roles in midgut remodeling by participating in the imaginal midgut formation.

Keywords *Helicoverpa armigera* · Rab32 · Midgut remodeling · 20E

The nucleotide sequence reported in this paper has been submitted to GenBank with accession number: HM037181.

Electronic supplementary material The online version of this article (doi:10.1007/s00726-010-0720-2) contains supplementary material, which is available to authorized users.

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Introduction

Insect molting and metamorphosis are complex physiological processes controlled by two hormones: ecdysone, and juvenile hormone (Riddiford 1993). Ecdysone initiates these processes by binding to the ecdysone receptor (EcR) to form an EcR/ultraspiracle protein (USP) complex. Then, it triggers the expression of downstream transcription factors, regulating effector gene expression (Hiruma and Riddiford 2001). Tissue remodeling occurs during insect metamorphosis. The old larval tissues decompose, and new adult tissues, such as the midgut and the fat body form (Parthasarathy and Palli 2007; Rabossi et al. 2004; Uwo et al. 2002).

The midgut undergoes remodeling during larval–pupal transition in most holometabolous insects. The replacement of the larval midgut during metamorphosis is regulated by 20E and JH. This phenomenon has been investigated in several insects, such as *Drosophila melanogaster*, *Aedes aegypti*, and *Heliothis virescens* (Cakouros et al. 2004; Nishiura et al. 2005). The process comprises a series of physiological events, including the disassembly of the old larval midgut and the formation of an imaginal midgut. In lepidopteran larvae, the larval midgut epithelium contains three cell types: columnar cells, goblet cells, and regenerative cells (Tettamanti et al. 2007a). During larval–pupal metamorphosis, the regenerative cells proliferate and differentiate into epithelium forming the pupal midgut (Parthasarathy and Palli 2008). The larval midgut condenses to the yellow body and decomposes, thus providing recycled materials for adult midgut formation (Hakim et al. 2009).

Both programmed cell death (PCD) and autophagy are involved in eliminating the old tissue during metamorphosis. Midgut remodeling depends on the combined

interaction of PCD and intense autophagy (Tettamanti et al. 2007b). Autophagy in the larval midgut requires the formation of vesicles shed from the midgut epithelium; these vacuoles may contain lysozymes to hydrolyze the old tissue to recycled materials. The imaginal midgut epithelium requires more ATP synthesis by mitochondria to absorb more nutrients, which in turn enhances the formation of mitochondria (Hakim et al. 2009). However, little is known regarding the factors that participate in nutrient absorption in the imaginal midgut.

Rab proteins belong to a subfamily of Ras GTPases that are able to bind and hydrolyze GTP to GDP. More than 60 Rab subfamily members have been identified in human (Pereira-Leal and Seabra 2001). The proteins are widely involved in various cellular functions, including membrane fusion and fission, exocytosis, endocytosis, vesicle trafficking, and cytoskeletal trafficking (Takai et al. 2001). New research has implicated Rab32 as a candidate binding protein in the biogenesis of lysosome-related organelles complex-1 (BLOC-1) (Rodriguez-Fernandez and Dell'Angelica 2009). BLOC-1 is a multi-subunit protein complex involved in intracellular membrane trafficking, biogenesis, and fusion of synaptic vesicles. It has been reported that Rab32 is related to mitochondrial fission. A recent study indicated that Rab32 is required to form autophagic vacuoles in HeLa cells (Hirota and Tanaka 2009).

Investigations of the role of Rab proteins in insect development have focused mostly on *Drosophila*. It was found that Rab11 participates in the development of nervous system during embryogenesis in *Drosophila* (Bhuin and Roy 2009a). Partial failure of myoblast fusion and anomalies in the shape of the muscle fibers have also been observed after the knockdown of Rab11 (Bhuin and Roy 2009b). Alone et al. found that Rab11 mutation causes eye defects during *Drosophila* eye development (Alone et al. 2005). Other research has revealed that Rab proteins could be phosphorylated by protein kinase C (PKC) in the brain of *Bombyx mori* (Uno et al. 2004). Recent findings have implied that Rab8 phosphorylation is involved in the prothoracicotropic hormone (PTTH) secretion in insects (Hiragaki et al. 2009).

However, the functions of Rab32 in insect development and tissue remodeling are unclear. We therefore investigated the role of Rab32 in the midgut remodeling of the cotton bollworm *H. armigera*, a lepidopteran insect, by studying its expression profile during development, hormonal regulation, and localization in the midgut, as well as the after effects of the knockdown of this gene on the imaginal midgut. All evidences obtained suggest that the gene is involved in imaginal midgut formation during metamorphic midgut remodeling.

Materials and methods

Insect rearing

The cotton bollworms were cultured in our laboratory at 27 ± 1°C under a photoperiod of 14 h light/10 h dark. The larvae were reared on an artificial diet described previously (Zhao et al. 1998).

HaRab32 full-length cDNA cloning

An expression sequence tag that encodes the 5′-end of *HaRab32* was obtained by random sequencing of the cDNA library of *H. armigera* larvae at the metamorphic stage. A gene-specific forward primer *HaRab32*F1 (5′-gct gagcgtcgggaacatc-3′) was designed based on the gene fragment. Both *HaRab32*F1 and the 3′-anchor primer (5′-gaccacgcgtatcgatgtcgac-3′) were used to amplify the 3′-end of the gene by rapid amplification of cDNA ends (3′-RACE). The full-length *HaRab32* cDNA was obtained by 5′-end and 3′-end primers of *HaRab32*EXPF (5′-ta ctcagaattcatgtcgcccaaacagaaagcc-3′) and *HaRab32*EXPR (5′-tactcactcgagtcagcatgagcatgactttgt-3′), from the start and the stop code of the cDNA. The polymerase chain reaction (PCR) procedure was as follows: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 53°C for 45 s, 72°C for 40 s, and 72°C for 10 min.

Phylogenetic analysis of *HaRab32*

Database searches were conducted using software BLASTX (http://www.ncbi.nlm.nih.gov/). Gene translation and protein prediction were conducted using ExPASy (http://www.au.expasy.org/). Domain prediction was undertaken using SMART software (http://smart.embl-heidelberg.de/). Sequence alignment and Phylogenetic tree analyses were conducted using the MEGA 3.1 software (http://www.megasoftware.net/) and the GENDOC computer program (http://www.nrbsc.org/downloads/gd322700.exe).

Expression pattern analysis of *HaRab32* by semi-quantitative RT-PCR

Total RNA was extracted from the cotton bollworm at different developmental stages with Unizol reagent (Biostar, Shanghai, China). The purified RNA samples were then digested with DNaseI to remove the genomic DNA. Of the total RNA, 5 μg was reverse transcribed into cDNA (FirstStrand cDNA Synthesis Kit, MBI Fermentas, St. Leon-Rot, Germany). The cDNAs were used as templates of RT-PCR. To determine the appropriate condition for

PCR amplification, the PCR templates were amplified for a different number of cycles from 20 to 33 (sampled every three cycles). The RT-PCR was performed under the following conditions: (94°C, 3 min); 27 cycles (94°C, 30 s; 55°C, 45 s; 72°C, 45 s); 1 cycle (72°C, 10 min). Primers used were *HaRab32*F, 5′-gctgagcgtcgggaacatc-3′; *HaRab 32*R, 5′-ctacaaggaggctgtgggag-3′. *Haβ-actinF*, 5′-cctggtatt gctgaccgtatgc-3′; *Haβ-actin*R, 5′-ctgttggaaggtggagagggaa-3′. The experiment was repeated three times using independent samples. The histograms were constructed according to the method of Sui et al. (2009).

Hormonal regulation

Ecdysone steroid 20-hydroxyecdysone (20E) and methoprene (Met) were first dissolved to 10 mg/ml in dimethyl sulfoxide (DMSO), then 1:100 diluted in PBS (140 mM NaCl, 10 mM sodium phosphate, pH 7.4). The accurate concentration was determined at OD 240 by calculating 1 mM = 12.4 OD. A solution of 5 µl (500 ng) 20E or methoprene was injected into the 6th 6 h instar larvae. The control panel was treated with an equal volume of DMSO. Total RNA was isolated from three to five larvae after treatment for 1, 3, 6, 12, and 24 h, and reverse transcribed into single-stranded cDNAs using M-MLV Reverse Transcriptase (Clontech, CA, USA).

Expression, purification, and antibody preparation of HaRab32

The ORF of HaRab32 was inserted into the pET30a(+) plasmid and sequenced to confirm it was in the frame of His-tag. The recombinant expression plasmid pET30a(+)-HaRab32 was transformed into competent *Escherichia coli* BL21 (DE3) cells. The recombinant protein was expressed in an insoluble form. The molecular weigh of the targeted protein was examined as 32 kDa by SDS-PAGE (HaRab32 26 kDa, the sequence including His-tag on the vector about 6 kDa). Buffer A [50 mM Tris–HCl (pH 8.0), 5 mM EDTA] and buffer B [50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 2 M urea] were used to wash the inclusion bodies. The protein band was gel-excised and dialyzed into running buffer electrically. Purified HaRab32 was used as an antigen to produce polyclonal rabbit antiserum using a method previously described (Wang et al. 2008). The specificity of the antibody was examined by immunoblotting analysis (Sup 1). Midguts of *H. armigera* at 6th instar 120 h (6th–120) were dissected and homogenized in PBS containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The homogenates were centrifuged at 10,000×*g* for 10 min at 4°C, and the supernatant was collected. The protein concentration was measured using the Bradford method (Bradford 1976). Western blot was carried out according to the method described by Yang et al. (2007).

RNA interference on HaEpi cell line

We have established HaEpi cell line in our previous work by Shao et al. (2008). So we investigated the regulation of HaEcRB1 on HaRab32 in HaEpi cells. For ds HaEcRB1 synthesis, DNA fragment of *HaEcRB1* was obtained by PCR amplification with specific primers (*HaEcRB1*RNAiF, 5′-gcgtaatacgactcactataggcgctggtataacaacggagga-3′ and *Ha EcRB1*RNAiR, 5′-gcgtaatacgactcactataggagctggagacaactcc tcacg-3′). PCR product extracted by phenol–chloroform method was used to synthesize *HaEcRB1* double-stranded RNA (dsRNA) using MEGAscript™ RNAi kit (Ambion, Austin, TX, USA). The HaEpi cells were incubated at 26°C in Grace's medium with 10% fetal bovine serum (FBS) to 80% confluence. The cells were then cultured in 4 ml Grace's medium without FBS containing 8 µg dsRNA and 10 µl Lipofectamine 2000 at 26°C for 12 h. Cells were re-fed with standard medium with 10% FBS containing 20E at a concentration of 0.4 µM. After 12 h of culturing, total RNA was extracted from cells for semi-quantitative RT-PCR analysis. Control cells were prepared using the same amount of dsGFP (GFPRNAiF, 5′-gcgtaatacgact cactataggtggtcccaattctcgtggaac-3′ and GFPRNAiR, 5′-gcgt aatacgactcactataggagctggagacaactcctcacg-3′). Detailed cell incubation and RNA interference methods were described in our earlier work (Shao et al. 2008).

Immunocytochemistry and immunohistochemistry

HaEpi cells were cultured in Grace's medium at 27°C for 72 h. The cells were treated with 1 μM 20E for 0.5 h and 6 h. DMSO was added to the control panel in the same volume. The incubated cells were fixed in 4% paraformaldehyde solution for 10 min at room temperature (RT), and then were washed with PBS for three times, and each for 10 min. The cells were permeabilized with PBS containing 0.2% Triton X-100 for 30 min. After blocking with 2% BSA for 30 min at 37°C, the cells were incubated with the antibody against HaRab32 (1:100 dilution). Goat anti-rabbit-Alexa Fluor 488 (diluted to 1:1,000) was used as a second antibody and incubated with the cells for 1 h at 37°C. Nuclei were stained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI; AnaSpec Inc., San Jose, CA; 1 µg/ml in water) for 10 min at RT. Negative control was incubated with pre-serum instead of the antibody against HaRab32.

Midguts of larvae at different developmental stages were dissected and fixed in 4% paraformaldehyde for 16 h at 4°C and then dehydrated and embedded in paraffin wax. Paraffin slices, 7 µm in thickness, were placed on glass

slides and dried overnight at 42°C. The slices were treated following the previously described method (Wang et al. 2007; Zhao et al. 2005). A primary antibody against HaRab32 was diluted to 1:100, and the goat anti-rabbit-ALEXA 488 (Eugene, USA) was diluted to 1:1,000 in 1× PBS with 2% BSA at RT for 2 h. For nuclei staining, DAPI (1 μg/ml in water) was incubated with the slides for 10 min. Negative controls were incubated with pre-immune rabbit serum instead of the antiserum against HaRab32. Fluorescence was observed with Olympus BX51 fluorescence microscope (Shinjuku-ku, Tokyo, Japan).

RNA interference by feeding bacterially expressed dsRNA on larvae

For RNAi in vivo, we constructed a plasmid expressing *HaRab32* dsRNA. A 580-bp DNA fragment of *HaRab32* was amplified by PCR (*HaRab32*RNAiF, 5′-tactca gcg gccgc ctgagcgtcgggaacatcta-3′ and *HaRab32*RNAiR, 5′-ta ctca ctcgag gcgaacttgtcaccatccct-3′). The PCR product was cut with *Not*I and *Xho*I, then inserted into plasmid L4440, which contains two convergent T7 polymerase promoters in opposite orientation separated by a multicloning site. The recombinant vector L4440-*HaRab32* was transformed into competent HT115(DE3) bacteria (both L4440 and HT115 were provided by Dr. Marek Jindra and Masako Asahina), which lacked RNaseIII and could be induced by β-D-thiogalactoside (IPTG). The bacteria could express dsRNA in the presence of 0.4 mM IPTG.

For dsRNA expression and feeding assay, we followed the methods of Fire and Tian (Tian et al. 2009; Timmons et al. 2001). The bacterial cells expressing dsRNA were grown to $OD_{600} = 0.4$ in 100 ml 2 × YT medium by 0.4 mM IPTG induction. The cells were collected and resuspended in 1 ml ddH_2O and used to feed the 3rd instar *H. amigera* larvae. A 50-μl suspension of the bacteria culture containing L4440-*HaRab32* was mixed with the artificial diet and the diet was cut into small pieces approximately 0.5 g in weight. The control group was covered by bacteria containing L4440-GFP (GFPERNAiF, 5′-tactca gcggccgc tggtcccaattctcgtggaac-3′ and GFPERNAiR, 5′-tactca ctcgag agctggagacaactcctcacg-3′) with the same amount of diet. All diets were replaced daily. The experiment was repeated three times. Total RNA was isolated from the 6th instar 72–96 h larvae and semi-quantitative RT-PCR was used to confirm the gene silencing effect in the experimental group and control group.

To determine the impact of *HaRab32* silencing on midgut remodeling, larvae from each group were individually selected for tissue structure observation. The midgut structure during metamorphosis was examined by immunohistochemistry and hematoxylin and eosin (H&E) staining.

Results

HaRab32 is a highly conserved gene

The entire sequence of *HaRab32* cDNA was 1,315 bp, and the open reading frame (ORF) encoded a 231-amino acid protein with a predicted molecular mass and isoelectric point of 25.9 kDa and 8.51, respectively (http://www.expasy.org/tools/protparam.html). The deduced protein had no secretory signal peptide but had a typical Rab32 and Rab38 subfamily domain spanning from residues 23 to 194 (Sup.2).

The multiple amino acid alignment of HaRab32 with other insects indicated that *Rab32* was a highly conserved gene (Sup.3A). The Phylogenetic analysis revealed that Rab32 and Rab38 were evolutionary relative, but our protein was homologous to human Rab32 (shown in Sup.3B). Cluster I included Rab32 proteins from insects and vertebrates, such as *A. aegypti*, *Culex quinquefasciatus*, and also vertebrates like *Danio rerio*, *Xenopus laevis* and *Homo sapiens*. Cluster II included Rab38 proteins from *Canis familiaris*, *Rattus norvegicus* and *H. sapiens*.

HaRab32 is highly expressed in the epidermis and midgut during metamorphosis

To understand the function of *HaRab32*, we first examined its expression patterns in various tissues during larval development. RT-PCR analysis revealed that *HaRab32* was expressed in various tissues including the epidermis, the midgut, and the fat body. *HaRab32* increased in certain tissues after the larvae entered metamorphosis. In the epidermis, the levels of *HaRab32* transcripts increased by 50% from the 6th instar 72 h larvae to the pupae. In the midgut, *HaRab32* was highly expressed from 6th 120 h larvae to the pupae. However, in the fat body, the expression was constantly high, and no such dynamic transcriptional regulation was detected during either molting or metamorphosis (Fig. 1). These data suggest that the expression of *HaRab32* in the epidermis and the midgut is correlated with metamorphosis.

HaRab32 is up-regulated by 20E

The expression pattern of *HaRab32* suggested that it might be regulated by 20E, because the known theory believes that 20E level was high and the JH was absent at the metamorphic stage. To confirm the hormone effect on *HaRab32*, we injected 20E or JH analog methoprene into the 6th instar larvae and analyzed the mRNA level of *HaRab32* in midgut. The results indicated that the mRNA level of *HaRab32* was up-regulated by 20E. It increased from 1 to 6 h after the injection of 20E; then the mRNA

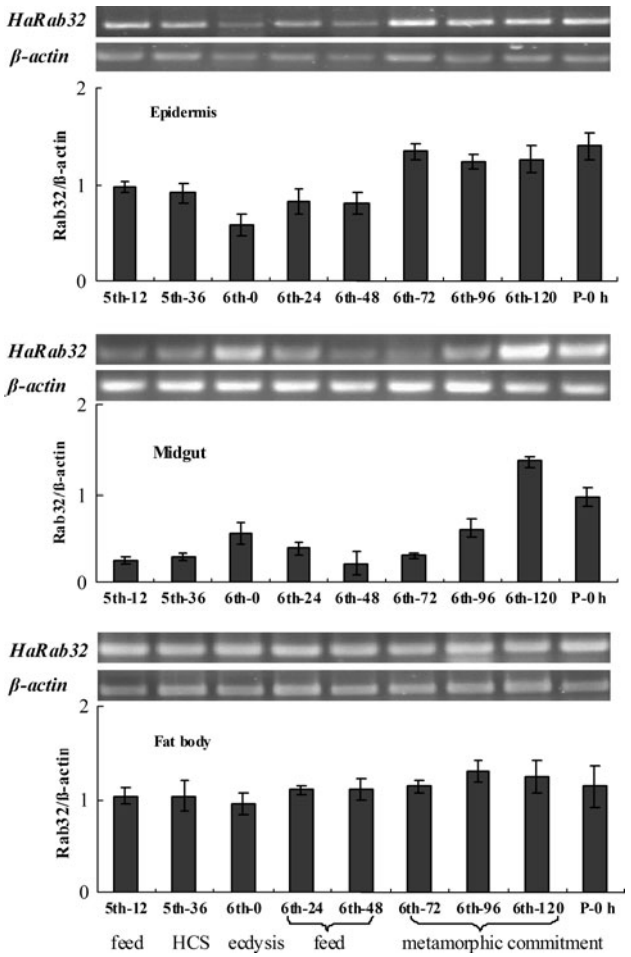

Fig. 1 RT-PCR analysis of the *HaRab32* expression in different tissues at various developmental stages. The 5th-12 to 6th-120 are larvae from 5th instar 12 h to 6th instar 120 h after ecdysis; *HCS* head capsule slippage; *P-0* the new pupae transformed from the last larval stage

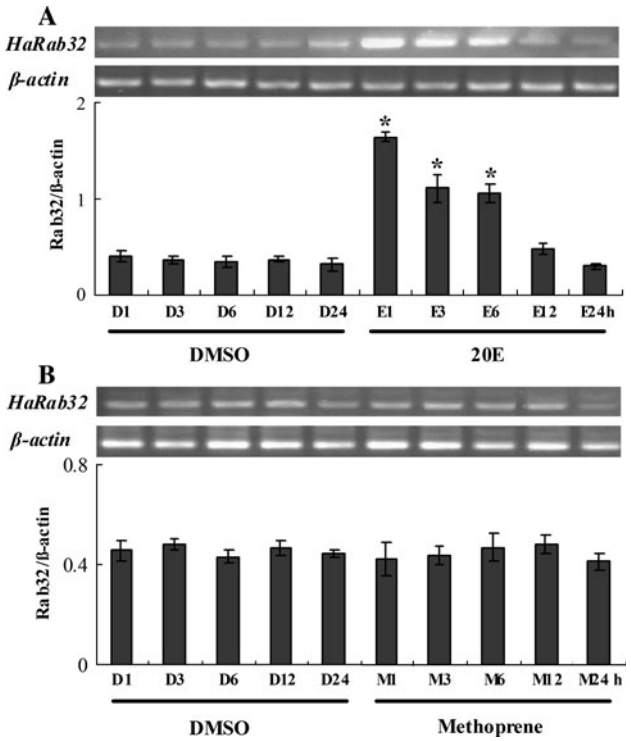

Fig. 2 Semi-quantitative RT-PCR to show the hormonal regulation on the expression of *HaRab32* in the midgut of the 6th instar 6 h larvae. **a** 20E treatment; **b** methoprene treatment. D1 to D24, E1 to E24, and M1 to M24 indicate 1, 3, 6, 12, and 24 h after treatments of equal amount of DMSO (as control), 20E, or methoprene in midgut. *Asterisks* indicate significant differences from treatment with DMSO alone ($p < 0.05$) by the Student's *t* test analysis

content decreased to its basal level (Fig. 2a). In contrast, the expression of this gene showed no obvious response to extra injected methoprene (Fig. 2b). These results indicate that *HaRab32* is regulated by 20E in this experimental condition.

20E regulates *HaRab32* expression through *EcRB1* in HaEpi cells

To examine if *HaRab32* was regulated by 20E through the ecdysone receptor *EcR* signal transduction pathway, we knocked down 20E-induced *HaEcRB1* expression in the epidermal cell line of HaEpi and examined its effect on *HaRab32* expression. The results show that the expression of *HaRab32* could not be induced by 20E after *HaEcRB1* knockdown, compared with the control in which both *HaEcRB1* and *HaRab32* could be up-regulated by 20E

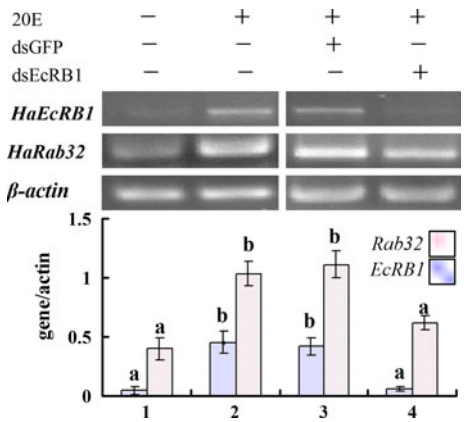

Fig. 3 Semi-quantitative RT-PCR to show the effect of knockdown *HaEcRB1* on *HaRab32* expression in HaEpi cells. Control was treated with the same amount of dsGFP. *β-actin* was used as a qualitative and quantitative control for RT-PCR. **a** and **b** show the significant difference, observed between the two columns of the same gene (*HaEcRB1* or *HaRab32*) by Student's *t* test analysis ($p < 0.05$)

(Fig. 3). This result indicates *HaRab32* is regulated by 20E signal transduction pathway through *EcRB1* as a receptor of 20E in HaEpi cells.

20E induced HaRab32 accumulation in the cytoplsma

To understand further the regulation of 20E on HaRab32 expression, immunocytochemistry was carried out to demonstrate whether 20E could influence the subcellular location of HaRab32 in HaEpi cells. The results showed that the basal HaRab32 signal mostly existed in the cytoplasm in normal condition. Immunocytochemistry and western blot showed that 20E did not cause translocation between cytoplasm and nuclei, but induced HaRab32 accumulation in the cytosolic granular astructures of unknown nature in HaEpi cells (Fig. 4). These results suggest that HaRab32 might play a special role by accumulating in some granules in the cytoplasm by 20E induction.

HaRab32 localized in the imaginal midgut

To understand the function of HaRab32 in the midgut, we investigated its localization in the midgut tissue. Results showed that the HaRab32 signal was detected in the cytoplasm of midgut cells in both the 5th feeding larvae and metamorphic committed larvae. In the 5th feeding larval midgut, the intensive signal was detected around the basement of the midgut and the apical region of the epithelium (Fig. 5a). In the metamorphic committed midgut at the 6th 120 h larval stage, the most intensive signal was detected at the apical region of the epithelium cells of the imaginal midgut (Fig. 5b).

Knockdown of *HaRab32* expression disrupts the imaginal midgut morphogenesis during metamorphosis

To understand further the roles of HaRab32 in the midgut, the *HaRab32* expression was knocked down by RNA interference via feeding the 3rd instar larvae with bacteria that expressed dsRNA of HaRab32. Semi-quantitative RT-PCR analysis showed that expression of *HaRab32* was successfully knocked down after feeding the bacteria that expressed dsRNA of *HaRab32* for 8 and 10 days when larvae were metamorphic committed at the 6th instar 72 and 96 h, respectively (Fig. 6a).

Considering that HaRab32 was highly expressed in the imaginal midgut, we examined the effect on the imaginal midgut by immunochemistry after the knockdown of *HaRab32* expression. Results showed that the protein level of HaRab32 decreased after the ingestion of dsHaRab32, which confirmed that the gene expression was knocked down by RNA interference via ingestion. Meanwhile, the morphology of the imaginal midgut was disordered in the *HaRab32* knockdown larvae (Fig. 6b).

To view clearly the structure of the imaginal midgut, we further examined the form of the imaginal midgut by HE staining. Pictures show that the midgut structure in the *HaRab32* expression knocked down larvae was less integral, while the epithelium cell layer was asymmetrical and not fully developed (Fig. 6c, dsGFP vs. dsRab32). This suggests the HaRab32 may be involved in imaginal midgut formation.

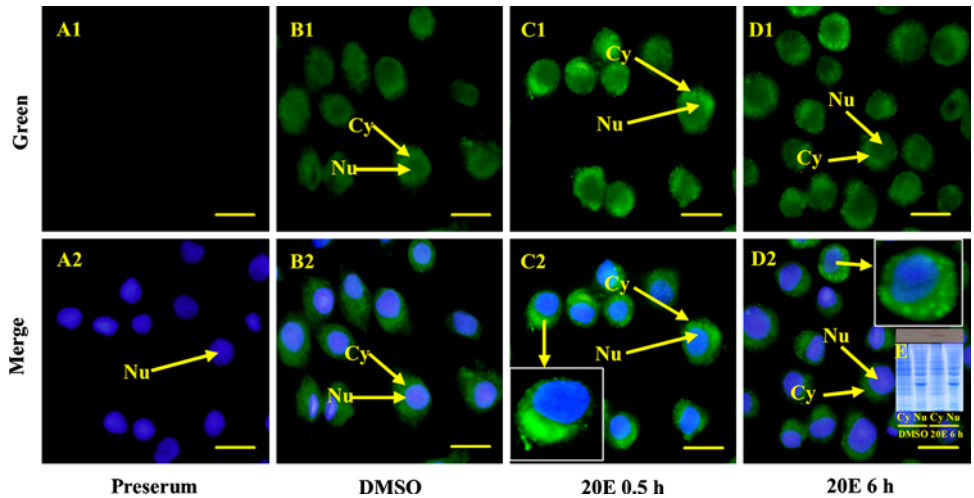

Fig. 4 Subcellular localization of HaRab32 in HaEpi cells by immunocytochemistry. *Nu* nucleus, *Cy* cytoplasm. *A1*, *B1*, *C1*, and *D1* indicate HaRab32 (*green*) stained for anti-HaRab32 and the second antibody marked with Alexa 488; *A2*, *B2*, *C2*, and *D2* show the HaRab32 merged with nucleus stained by DAPI. *Panels A1* and *A2* are negative controls using pre-antiserum; *B1* and *B2* are controls using equal amount of DMSO as the solvent for 20E; *C1* and *C2*, and *D1* and *D2* are treated with 20E for 0.5 and 6 h, respectively. *E* is western blot analysis the distribution of HaRab32 in cytoplasm and nucleus. *Bar* 50 μm (color figure online)

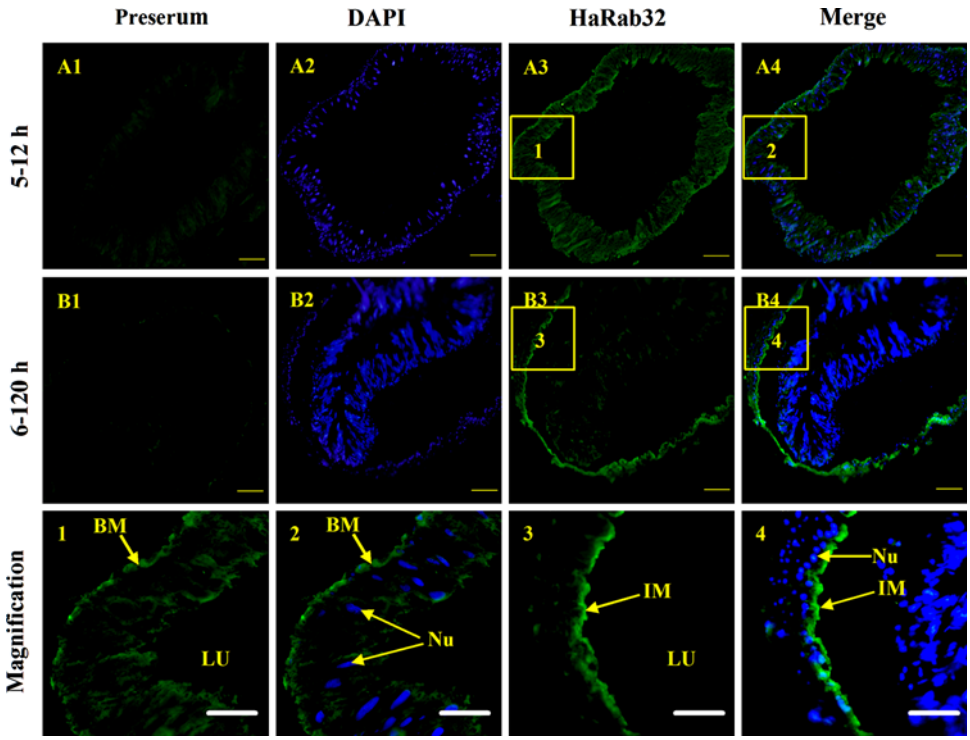

Fig. 5 Localization of HaRab32 in the midgut. *Nu* nucleus, *Cy* cytoplasm, *BM* basement, *LU* lumen, *LM* larval midgut, *IM* imaginal midgut. Cross-sections of the midgut from 5th–12 h (*A1–A4*) and 6th–120 h (*B1–B4*) larvae. *Panels A1* and *B1* are negative controls with pre-serum; *A2* and *B2* are DAPI staining to show the nucleus; *A3* and *B3* indicate HaRab32 (*green*) stained for anti-HaRab32 and secondary antibodies with Alexa 488; *A4* and *B4* are merged pictures of HaRab32 (*green*) and DAPI staining; *1*, *2*, *3*, and *4* are *magnified squares* in *A3*, *A4*, *B3*, and *B4*, respectively. *Scale bar* 100 mm (*yellow*), 25 mm (*white*) (color figure online)

Discussion

HaRab32 was up-regulated in the midgut during metamorphosis. It was located in the basement and the apical areas of the feeding larval midgut and the apical area of the imaginal midgut epithelium of the metamorphically committed larvae, respectively. The results suggest that *HaRab32* may play special roles in the midgut.

The apical region of the midgut could be involved in secretion of peritrophic membrane (Harper and Hopkins 1997; Ryerse et al. 1994), endocytosis of transcellular vesicles (Casartelli et al. 2005, 2007), recycling of membrane due to the constant endocytotic activity in apical region, and possibly signal transduction due to the stimulants in the lumen of the midgut arrives at the apical region first. The 20E treatment induced aggregation of HaRab32 to granules in the cytosol in the HaEpi cells may refer to autophagy vacuoles in human Hela cells (Hirota and Tanaka 2009). However, the disrupted formation and the irregular epithelium configuration of imaginal midgut after knockdown of *HaRab32* expression by RNAi suggest that HaRab32 participates in the midgut remodeling during metamorphosis, and the possible mechanism is that HaRab32 may relate to the cytoskeleton organization of imaginal midgut epithelium. Rab protein has been found participating in the remodeling of actin cytoskeletons (Nishimura and Sasaki 2009). The higher-level expression of *HaRab32* in the epidermis at the metamorphic stage may be involved in epidermis remodeling.

The basement area of the larval midgut includes muscles, basement membrane, and regenerative cells from the hemocoel to the gut lumen. The basal region of the midgut could be involved in muscle attachment, exocytosis of transcellular vesicles (Casartelli et al. 2005, 2007), and possibly signal transduction from the hemolymph. According to the location of HaRab32 in the basement area of the feeding larval midgut, HaRab32 is likely to relate to cellular adhesion in the midgut, which facilities the cells attaching to the muscles. Cell adhesion between cells and extra cellular matrix is important for cell morphogenesis (Nishimura and Sasaki 2008). Accumulating evidences have found that Rab proteins are involved in the regulation of epithelial junctions (Nishimura and Sasaki 2009).

Hakim et al. (2009) reported that the larval midgut is digested by apoptosis or autophagy programmed cell death during metamorphosis, and the imaginal midgut absorbs the nutrients provided by the hydrolyzed larval midgut to build a new midgut. Previous researches also demonstrated that Rab proteins played a major role in membrane fission/fusion and transport that could be related to nutrient uptake or membrane recycle (Jordens et al. 2005). So another possible function of HaRab32 during metamorphosis is that this protein is involved in the vesicle fusions, which probably associate with absorption of nutrients from the digested larval midgut for new midgut formation.

However, the knockdown of *HaRab32* expression had no obvious influence on larval growth and metamorphosis. The interfered larvae could grow and transform to pupae

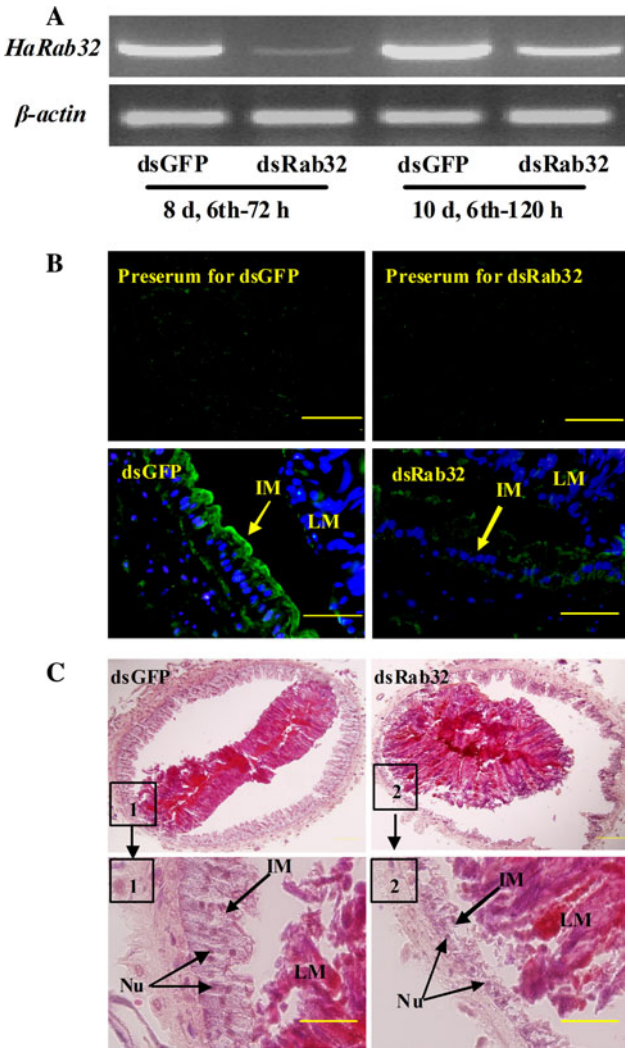

Fig. 6 The effect of dsHaRab32 on the imaginal midgut formation. **a** Semi-quantitative RT-PCR to show the knockdown of *HaRab32* after ingestion of bacteria that express dsRNA of *HaRab32*. Total RNA was extracted from four larvae after feeding the bacteria for 8 and 10 days at 6th–72 h and 6th–120 h, respectively, dsGFP-expressed bacteria were used as a non-specific dsRNA feeding control. *β-actin* was used as a reference. **b** Fluorescence immunochemistry to show the impact of dsHaRab32 on the imaginal midgut in the metamorphically committed larvae. *IM* imaginal midgut. DAPI staining to show the nucleus; HaRab32 (*green*) stained by anti-HaRab32 and Alexa 488; *Scale bar* 50 μm (*long line*), 200 μm (*short line*). **c** H&E staining immunochemistry to show the structures of the midguts from knockdown of *HaRab32* larva and the control of dsGFP-expressed bacteria feeding larva (color figure online)

normally. This is understandable because *HaRab32* is expressed greatly in the imaginal midgut, but not in the larval midgut. Therefore, the knockdown of this gene does not obviously disturb larval growth and pupation because the imaginal midgut is responsible for pupal or adult development. If there is any effect on the reproduction of the adult after knockdown of *HaRab32* expression needs further study.

HaRab32 was strongly up-regulated by 20E, indicating that *HaRab32* is regulated by the 20E signal transduction pathway. The suppression of the *HaRab32* expression after *HaEcRB1* expression knockdown indicated that *HaRab32* is regulated by the 20E hormone through 20E hormone receptor EcRB1 in HaEpi cells. According to our results, *HaRab32* is involved in midgut remodeling by participating in the imaginal midgut formation under the regulation of ecdysone hormone.

Acknowledgments This work was supported by grants from the National Basic Research Program of China (2006CB102001) and the National Natural Science Foundation of China (No. 30710103901). We thank Dr. Marek Jindra and Masako Asahina in Biology Center, Czech Academy of Sciences and Department of Molecular Biology, University of South Bohemia, Czech Republic, for providing the plasmid L4440 and the HT115(DE3).

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